

Understanding the Mechanisms Controlling *Leishmania amazonensis* Infection In Vitro: The Role of LTB₄ Derived From Human Neutrophils

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Neutrophils are rapidly recruited to the site of *Leishmania* infection and play an active role in capturing and killing parasites. They are the main source of leukotriene B₄ (LTB₄), a potent proinflammatory lipid mediator. However, the role of LTB₄ in neutrophil infection by *Leishmania amazonensis* is not clear. In this study, we show that *L. amazonensis* or its lipophosphoglycan can induce neutrophil activation, degranulation, and LTB₄ production. Using pharmacological inhibitors of leukotriene synthesis, our findings reveal an LTB₄-driven autocrine/paracrine regulatory effect. In particular, neutrophil-derived LTB₄ controls *L. amazonensis* killing, degranulation, and reactive oxygen species production. In addition, *L. amazonensis* infection induces an early increase in Toll-like receptor 2 expression, which facilitates parasite internalization. Nuclear factor kappa B (NFκB) pathway activation represents a required upstream event for *L. amazonensis*-induced LTB₄ synthesis. These leishmanicidal mechanisms mediated by neutrophil-derived LTB₄ act through activation of its receptor, B leukotriene receptor 1 (BLT1).

Keywords. *Leishmania amazonensis*; LPG; human neutrophils; leukotriene B₄; TLR2; NFκB.

Leishmaniasis represents a group of diseases that are a health problem in 90 countries, with 2 million new cases per year [1]. *Leishmania amazonensis* belongs to the *Leishmania mexicana* complex, which comprises species of cutaneous forms in the New World. In South America, *L. amazonensis* can cause severe

cutaneous leishmaniasis that is characterized by multiple nodules, known as diffuse cutaneous leishmaniasis [2].

Leishmania sp. interact with and infect different cell types [3]. Studies in mice showed that neutrophils are predominantly recruited and infected at early stages of infection in the skin [4]. Intravital imaging provides a visual demonstration of the rapidity of infection, with neutrophils descending on *Leishmania major* after transmission [5]. The engulfment of *L. major* by human neutrophils prevents fusion with primary and tertiary granules, which contain the machinery necessary for reactive oxygen species (ROS) generation and acidification, which are critical processes for parasite survival [6].

The major function of neutrophils is phagocytosis and killing of microorganisms through ROS generation and release of granule enzymes [7]. As neutrophils proceed through the endothelium, tertiary granules are mobilized and metalloproteases are released, which help neutrophils traverse the basement membrane and

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extracellular matrix [8]. At the inflammatory site, complete activation of neutrophils prompts the initiation of the oxidative burst and the mobilization of primary granules, which contain myeloperoxidase (MPO), a critical enzyme for the oxidative burst [9].

Lipid mediators are important players during the early immune response against *Leishmania* [10, 11] and contribute to parasite establishment or destruction. Products derived from 5-lipoxygenase (5-LO) are released during the infection of mouse macrophages by *Leishmania donovani* [12] and *L. amazonensis*, and leishmanicidal activity induced by leukotriene B₄ (LTB₄) in mouse macrophages is dependent on nitric oxide production [10]. Signaling through LTB₄ induces leukocyte accumulation [13], microbial ingestion [14], and killing [15]. LTB₄ also plays a critical role in human neutrophil migration induced by Toll-like receptor (TLR) stimulation [16]. TLR ligands prime LTB₄ production and neutrophil migration [17], and the LTB₄ receptor, B leukotriene receptor 1 (BLT1), is responsible for mediating these effects [18].

Although the effect of LTB₄ on the leishmanicidal activity of macrophages was previously described, this effect in human neutrophils, which is the major source of this lipid mediator, has not been addressed. Therefore, here, we characterize the profile and function of human neutrophils infected with *L. amazonensis*. We also investigate the role of TLR and BLT1 signaling in LTB₄ production, including the pathways involved and their role in parasite control.

METHODS

Ethics

Blood sample collection from subjects was performed after obtaining informed consent, in accordance with the Declaration of Helsinki, and approval from the ethical review board.

Neutrophil Culture and Infection

Human blood was obtained from healthy donors at the Hemocentro do Estado da Bahia, BA, Brazil. Neutrophils were isolated by centrifugation using Polymorphprep medium according to the manufacturer's instructions (AXIS-SHIELD). Polymorphonuclear cells were collected and washed 3 times at 4°C at 200 g. These cells were then labeled with human anti-CD16 antibody and analyzed by flow cytometry (see [Supplementary Figure 1](#)). Neutrophils were infected or not with *L. amazonensis*-Green fluorescent protein (GFP) (MHOM/BR/87/BA125, a stable transfected line expressing GFP, at a 1 cell to 10 *L. amazonensis* ratio) or its lipophosphoglycan (LPG; 5 µg/mL) for 3 hours. The infection rate was evaluated by optical microscopy after hematoxylin-eosin staining or by flow cytometry. Representative images were obtained using optical or confocal microscopy (see [Supplementary Figure 2](#)) after diamidino-2-phenylindole staining (Vector Laboratories).

Parasite Culture and Viability

Leishmania amazonensis metacyclic promastigotes were obtained from stationary-phase cultures (5–7 days). After 3 hours of culture with neutrophils, cells were washed twice for 5 minutes at 100 g and fed with supplemented Schneider's medium. The cells were then cultured at 23°C for an additional 24 hours. The relative intracellular load of *L. amazonensis* was measured by assessing the number of extracellular motile promastigotes produced [19]. The profiles of relative parasite loads closely followed the microscopic assessment of the number of internalized parasites per 100 neutrophils and the percentage of infected cells.

LPG Extraction and Purification

LPG was extracted from stationary promastigotes in solvent E (water/ethanol/diethyl ether/pyridine/ammonium hydroxide; 15:15:5:1:0.017). The extract was dried by nitrogen gas evaporation, resuspended in 0.1 N acetic acid/0.1 M sodium chloride, and applied to a column of phenyl-Sepharose (2 mL). LPG was eluted using solvent E [20]. The purity of LPG was previously tested using Chinese hamster ovary cells transfected with TLR2 or TLR4 and CD25 as a reporter protein [21].

Antibodies and Reagents

Single-cell suspensions were stained with phycoerythrin-conjugated CD11b, CD18, CD62L, CD16, TLR2, TLR4, and isotype control antibodies (BD Biosciences). Dihydroethidium was used to detect ROS (10 µM; Invitrogen). In some cases, neutrophils were treated for 15 minutes with inhibitors and washed twice (5 minutes at 100 g) prior to infection. These inhibitors included Zileuton (1 µM, 10 µM, and 100 µM; Cayman), tissue inhibitor of metalloproteinase 1 (TIMP-1; 30 ng/mL; Calbiochem), anti-MPO (1 µg/mL; R&D), antineutrophil elastase (anti-NE; 50 µg/mL; Calbiochem), anti-TLR2 and anti-TLR4 (100 µg/mL; InvivoGen), PI3k inhibitor (LY294002; 10 µM; Cayman), extracellular signal-regulated kinase (ERK) kinase inhibitor (PD98059; 50 µM; Promega), protein kinase C (PKC) inhibitor bisindolylmaleimide (BIS; 20 nM; Cell Signaling Technology), five lipoxygenase activating protein (FLAP) inhibitor MK886 (10 µM; Cayman), I kappa B alpha kinase phosphorylation inhibitor 3-[(4-methylphenyl)sulfonyl]-(2E)-propenenitrile (BAY)-11-7082 (10 µM; Calbiochem), BLT1 antagonist CP105969 (100 nM, 1 µM, and 10 µM; Pfizer), and IκB kinase complex inhibitor Wedelolactone (80 µM; Sigma). The concentrations used were based on previous studies [22]. For add-back experiments, exogenous LTB₄ (10 pg/mL and 50 pg/mL; Cayman) was added to the culture 30 minutes prior to infection, according to the method described in previous studies [15]. These concentrations were used based on the level of LTB₄ production by resting and infected neutrophils. Control cells were treated with medium containing the vehicle dimethyl sulfoxide (DMSO; ACROS).

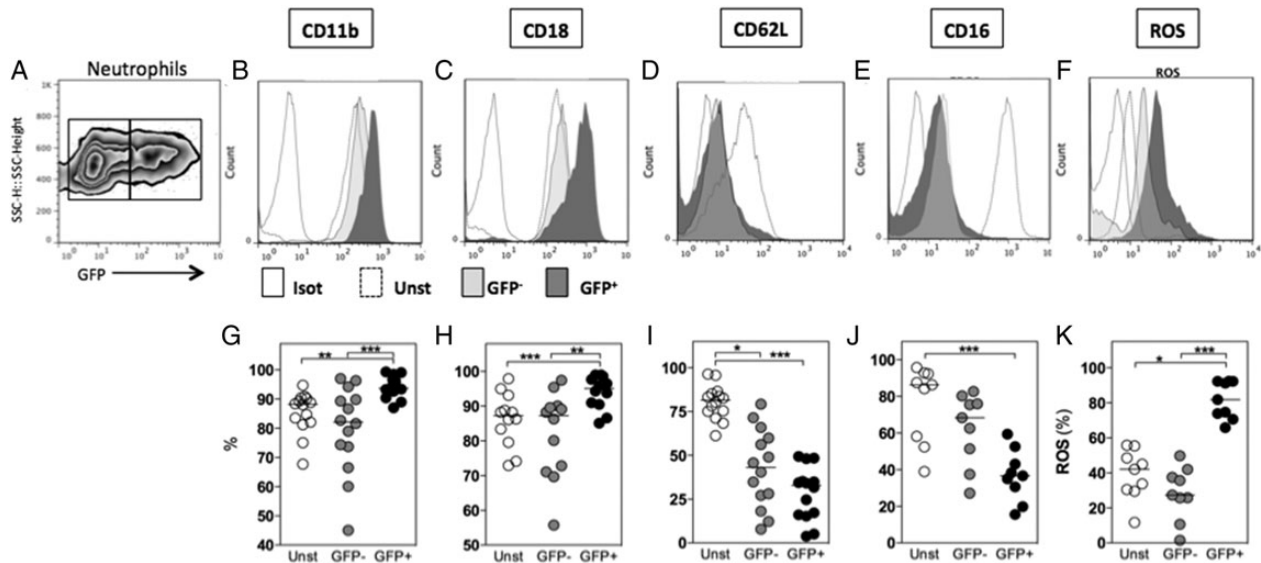


Figure 1. The interaction between human neutrophils and *Leishmania amazonensis* induces the differential expression of surface markers and reactive oxygen species (ROS) production. Human neutrophils were cultured alone (unstimulated) or infected with *L. amazonensis*–Green fluorescent protein (GFP) (1 neutrophil to 10 *L. amazonensis*) for 3 hours. The mean intensity fluorescence (B–F) and the frequency of cells expressing surface markers (G–K) were compared among unstimulated, noninfected bystander (GFP⁻), and infected (GFP⁺) cells (A). The expression of surface markers, such as CD11b (B and G), CD18 (C and H), CD62L (D and I), and CD16 (E and J), as well as ROS production (F and K) were evaluated by flow cytometry. **P* ≤ .05; ***P* ≤ .01; ****P* ≤ .001.

Measurement of Mediator Production

After 3 hours of culture, the supernatants were collected and immediately used to measure MPO and NE activity following a previously published protocol [19]. After 10–20 minutes, the reaction was stopped with 50 μL 8 N sulfuric acid. MPO activity was measured by reading the absorbance at 492 nm. For NE activity, the absorbance was measured at 410 nm, using a dose curve of serial dilutions of purified human NE starting at 1 U/mL. LTB₄ and prostaglandin E₂ were measured using enzyme-linked immunoassay kits (Cayman). Tumor necrosis factor-α (TNF-α) was measured by sandwich enzyme-linked immunosorbent assay (BD Bioscience).

Statistical Analysis

Data were analyzed using GraphPad Prism 5.0 software. The results were analyzed using the Mann–Whitney *t* test or Kruskal–Wallis test with Dunns post test and are expressed as median values.

RESULTS

L. amazonensis Infection Induces the Expression of Surface Markers and ROS Production in Human Neutrophils

Human neutrophils were infected with *L. amazonensis*–GFP; 3 hours later, the infection rate was evaluated. No differences were observed in the frequency of infected cells as evaluated by optical microscopy and flow cytometry (see [Supplementary Figure 2A](#)). Cells cultured in medium alone (resting neutrophils)

were termed “unstimulated.” Cells cultured with *Leishmania* could be infected or not. GFP⁻ cells were cultured with *Leishmania*–GFP but were not fluorescent by flow cytometry analysis. GFP⁻ cells were assumed to be uninfected, whereas GFP⁺ cells were considered infected (Figure 1A). The mean fluorescence intensity (MFI) and the frequency of cells expressing surface markers were compared among unstimulated, GFP⁻ (bystander), and GFP⁺ (infected) cells. The MFI of CD11b (Figure 1B) and CD18 (Figure 1C) was upregulated in GFP⁺ cells, and similar results were obtained for the percentage of cells expressing these markers (Figure 1G and 1H). In comparison to unstimulated cells, *L. amazonensis* infection decreased the MFI and frequency of GFP⁺ cells expressing CD62L (Figure 1D and 1I) and CD16 (Figure 1E and 1J), although no differences were observed between GFP⁺ and GFP⁻ cells, indicating that bystander cells presented a similar degree of activation. Cells were also evaluated for ROS production, and the MFI was increased in GFP⁺ cells (Figure 1F). Approximately 80% of GFP⁺ cells produced ROS compared with 38% of unstimulated or GFP⁻ cells (Figure 1K). These results indicate that *L. amazonensis* infection induces the activation of human neutrophils.

Lipid Mediator Production and Neutrophil Degranulation Are Induced Following Exposure to *L. amazonensis* or LPG

Following *L. amazonensis* infection, a significant increase in LTB₄ production by human neutrophils was observed (Figure 2A). However, no differences in the production of TNF-α or prostaglandin E₂ were observed between unstimulated and

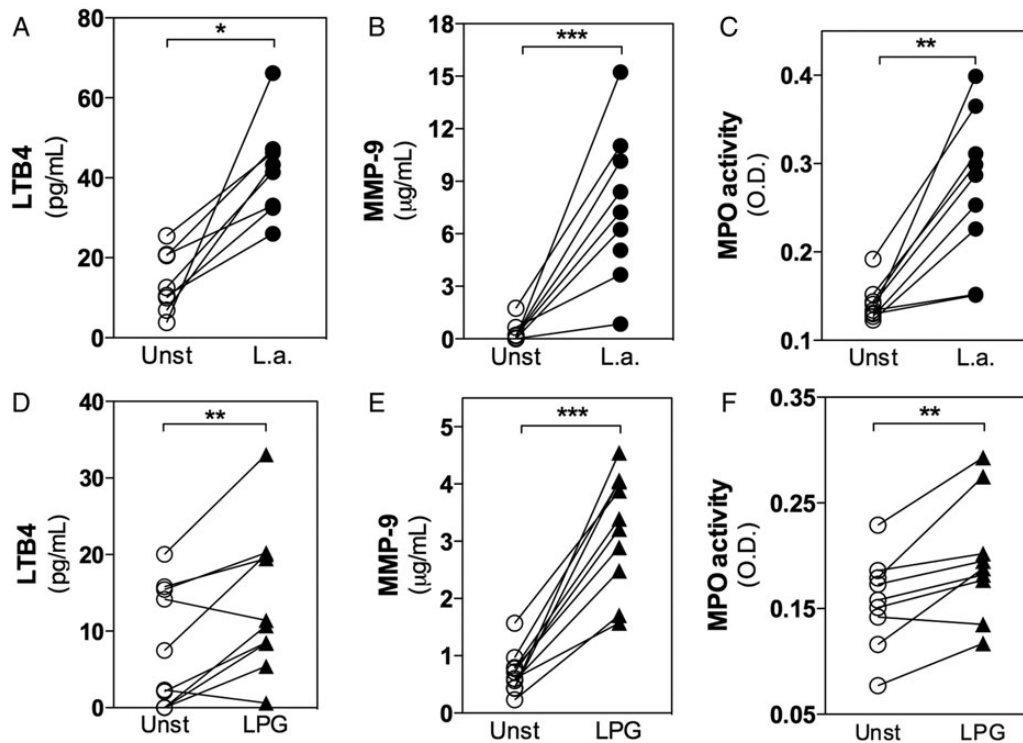


Figure 2. *Leishmania amazonensis* infection or lipophosphoglycan (LPG) triggers leukotriene B₄ (LTB₄) production and the release of granule content from human neutrophils. Neutrophils were cultured alone (unstimulated [unst]), infected with *L. amazonensis*-GFP (1 neutrophil to 10 *L. amazonensis*; A–C), or treated with LPG (5 μg/mL) for 3 hours (D–F). Supernatants from the cultures were used to measure LTB₄ production by competition enzyme-linked immunosorbent assay (ELISA; A and D) and metalloproteinase-9 (MMP-9) by sandwich ELISA (B and E). Myeloperoxidase (MPO) was evaluated according to its activity for a specific substrate (C and F). **P* ≤ .05; ***P* ≤ .01; ****P* ≤ .001.

infected neutrophils (data not shown). To evaluate degranulation, the metalloproteinase-9 (MMP-9) concentration was measured in order to evaluate the release of tertiary granules. The activity of enzymes present in primary granules, such as MPO and NE, was also evaluated. The levels of these enzymes were significantly increased in supernatants from infected neutrophils, indicating that *L. amazonensis* infection induced the release of tertiary (Figure 2B) and primary granules (Figure 2C).

LPG represents a main virulence factor associated with *Leishmania* infection [21], and neutrophil treatment with *L. amazonensis* LPG induced a response that was similar to that observed for the whole parasite. LTB₄ production (Figure 2D), MMP-9 release (Figure 2E), and MPO activity (Figure 2F) were also increased in neutrophils treated with LPG. These data support the activation of human neutrophils by *L. amazonensis*, as observed through the expression of surface molecules, ROS production (Figure 1), and degranulation (Figure 2).

TLR2 Plays an Active Role in *L. amazonensis* Internalization by Human Neutrophils

TLRs detect pathogen-associated molecular patterns, such as lipoproteins, from viruses, bacteria, fungi, and parasites [23]. Since *L. amazonensis* and LPG induced similar responses in neutrophils (Figure 2), the expression of TLRs was evaluated.

There was an increase in the expression of TLR2 (Figure 3A) and TLR4 (Figure 3B) at 30 minutes post-infection in GFP⁺ cells. However, at 3 hours post-infection, only TLR2 expression was significantly reduced to a basal level (Figure 3A). These data suggest that TLR2 is internalized along with *L. amazonensis* and that this process may be mediated by LPG recognition. In contrast, no difference in TLR4 expression was observed between 30 minutes and 3 hours post-infection (Figure 3B), suggesting that TLR4 was not involved.

To confirm whether TLR2 was internalized with the parasite, we quantified the infection rate and the number of internalized parasites in neutrophils treated with neutralizing antibodies against TLR2 and TLR4. In the absence of TLR2, the infection rate (Figure 3C) and the number of internalized parasites (Figure 3D) were reduced, whereas no differences were observed following treatment with anti-TLR4 antibody (Figure 3C and 3D).

Inhibition of 5-LO Increases Human Neutrophil Susceptibility to *L. amazonensis* Infection

The role of lipid mediators in the control of *L. amazonensis* was investigated by treating neutrophils with pharmacological inhibitors of 5-LO prior to infection. The following concentrations of Zileuton, a 5-LO specific inhibitor, were tested: 1 μM, 10 μM, and 100 μM. Treatment with Zileuton at 1 μM did not

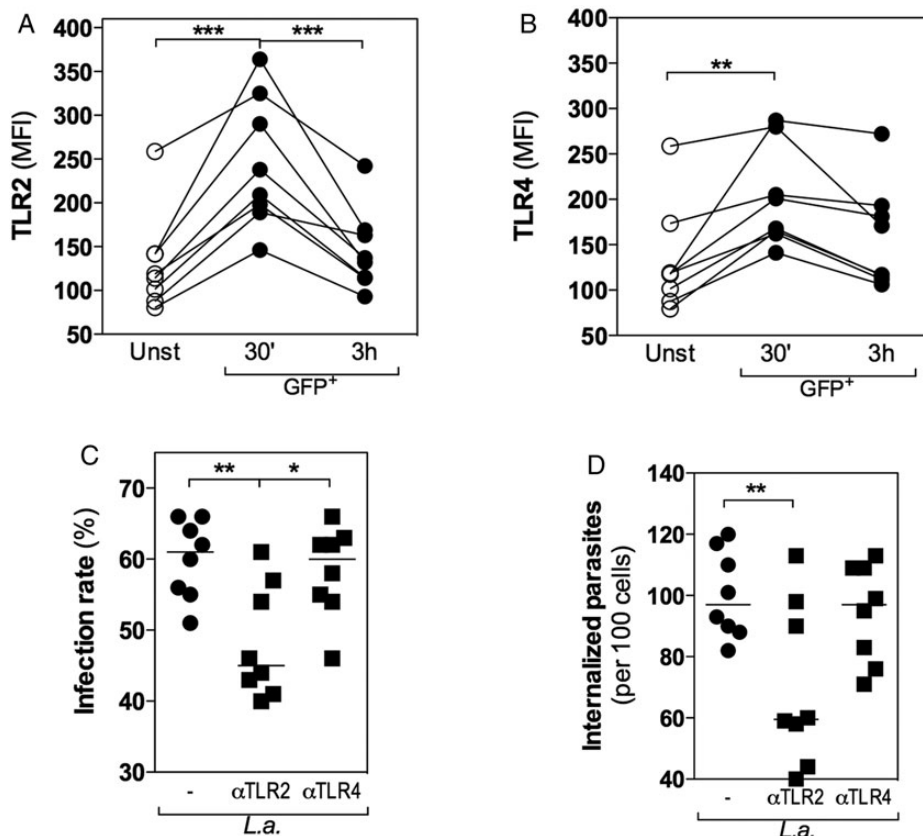


Figure 3. Toll-like receptor 2 (TLR2) plays an active role in *Leishmania amazonensis* internalization by human neutrophils. Neutrophils were cultured alone (unstimulated [unst]) or treated with neutralizing antibodies against TLR2 and TLR4 (100 µg/mL) for 15 minutes prior to infection with *L. amazonensis*-GFP (1 neutrophil to 10 *L. amazonensis*). The kinetics of TLR2 (A) and TLR4 (B) expression were compared by flow cytometry for unstimulated and GFP⁺ cells after 30 minutes and 3 hours of infection. The percentage of infected cells (C) and the number of internalized parasites (D) were quantified by optical microscopy after 3 hours of infection. * $P \leq .05$; ** $P \leq .01$; *** $P \leq .001$.

produce any effect on parasite viability in infected neutrophils. The number of live *Leishmania* 24 hours after treatment was similar to that of control cells cultured in the absence of Zileuton. However, treatment of infected neutrophils with 10 µM and 100 µM Zileuton significantly increased parasite viability with similar magnitude (data not shown). Based on these data, 10 µM was chosen as the optimal concentration to evaluate 5-LO inhibition. Zileuton (Figure 4A) and MK886, a FLAP inhibitor (Figure 4B), induced a significant increase in parasite viability, and similar results were obtained with MMP-9 and MPO inhibitors (TIMP-1 and αMPO, respectively). However, the NE inhibitor had no effect on parasite viability (see Supplementary Figure 3).

Effects of LTB₄ Are Mediated Through Its High-Affinity Receptor BLT1

To evaluate the role of LTB₄/BLT1 signaling in the control of *L. amazonensis* infection, an antagonist of BLT1, CP105696 (CP), was used. The increased parasite viability observed following CP treatment (Figure 4C) confirmed the importance of this

pathway for parasite killing. Furthermore, the addition of exogenous LTB₄ to cultures containing Zileuton-treated neutrophils restored their ability to control *L. amazonensis* (Figure 4D).

By transmission electron microscopy, unstimulated neutrophils presented a normal shape, with cytosolic granules and multilobulated nuclei (data not shown). In Figure 4E, the *Leishmania* inside the parasitophorous vacuoles (delimited by white lines) presents ultrastructural abnormalities, such as condensed chromatin, vacuole-like structures, and poorly preserved cytoplasm and organelles, indicating parasite killing. Conversely, in Zileuton-treated infected neutrophils, the parasites appeared viable, with intact kinetoplasts and flagella (Figure 4F), suggesting that leukotrienes play a significant role in *Leishmania* elimination.

Degranulation and TLR2 Expression in Human Neutrophils Infected With *L. amazonensis* Are Partially Dependent on LTB₄

LTB₄ can lead to neutrophil degranulation [18], and significant reductions in MMP-9 concentration and MPO activity were observed when cells were treated with Zileuton (Figure 5A and 5B). These results suggest that LTB₄ production in neutrophils

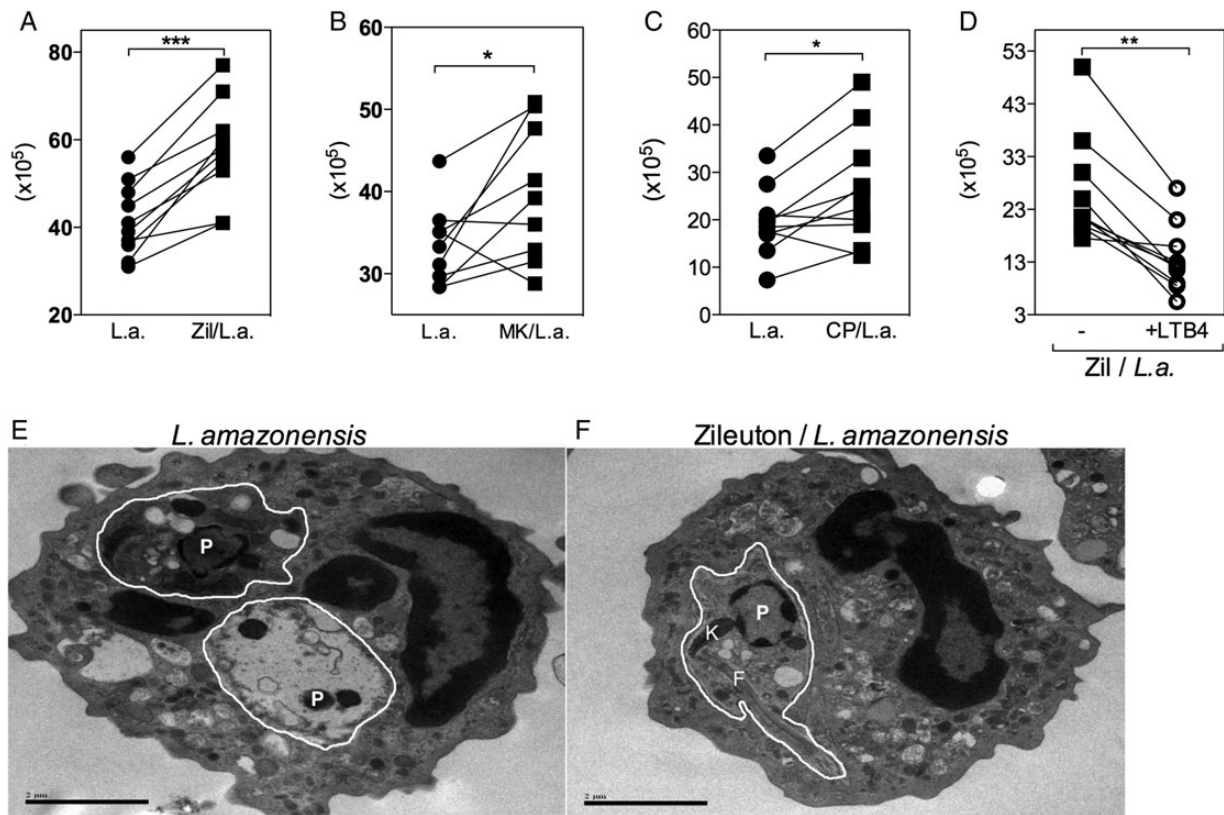


Figure 4. Inhibition of 5-lipoxygenase increases human neutrophil susceptibility to *Leishmania amazonensis* infection through the high-affinity leukotriene B₄ (LTB₄) receptor B leukotriene receptor 1 (BLT1). Human neutrophils were treated with 10 μM Zileuton (A), 10 μM MK886 (B), or 100 nM CP105969 (C) for 15 minutes prior to infection with *L. amazonensis*-GFP (1 neutrophil to 10 *L. amazonensis*). Exogenous LTB₄ was added to selected cultures of Zileuton-treated neutrophils (D). After 3 hours of infection, cells were washed, fed with supplemented Schneider's medium, and cultured at 23°C for an additional 24 hours. Parasite viability was measured by assessing the number of extracellular motile promastigotes produced. Transmission electron microscopy was used to investigate ultrastructural morphological alterations in infected (E) and Zileuton-treated infected neutrophils (F). White lines delimit parasitophorous vacuoles. Parasites (P), kinetoplasts (K), and flagella (F) are indicated. **P* ≤ .05; ***P* ≤ .01; ****P* ≤ .001.

is induced following *L. amazonensis* infection, leading to degranulation. ROS represent a well-established mediator of leishmanicidal mechanisms, and their level of production was also reduced in GFP⁺ cells pretreated with Zileuton (Figure 5C). These results reinforce the essential role of leukotrienes in the early events of degranulation cascade, including the release and activity of several mediators crucial for parasite control.

The expression of TLR2 and TLR4 in GFP⁺ neutrophils treated with Zileuton was also compared, and this treatment significantly reduced the expression of TLR2 (Figure 5D), but not TLR4 (Figure 5E), after 30 minutes in GFP⁺ cells. Furthermore, our results showed a significant reduction in the internalization of *L. amazonensis* by Zileuton-treated neutrophils (Figure 5F). These data reinforce the role of LTB₄ in TLR expression and its recognition of pathogen-associated molecules. In addition, parasite killing was increased when infected neutrophils were treated with Pam3CSK4 (a TLR2 agonist) during the infection (data not shown), indicating that further activation of TLR signaling likely stimulates the mechanisms involved in parasite control.

Intracellular Signaling Pathways Regulate LTB₄-Mediated Leishmanicidal Activity in Human Neutrophils

The responses initiated by TLRs culminate in Nuclear factor kappa B (NFκB) and mitogen-activated protein kinase (MAPK) activation [24]. BAY, a specific inhibitor of NFκB, which prevents the phosphorylation of IκB, significantly increased the number of viable parasites, suggesting a reduction in leishmanicidal activity (Figure 6A). Similar results were obtained with Wedelolactone, a nonspecific NFκB inhibitor (data not shown). The number of viable parasites was also increased in cultures of infected neutrophils treated with LY294002 (LY), a PI3k inhibitor (Figure 6B); PD98059 (PD), an ERK inhibitor (Figure 6C); and BIS, an adenosine triphosphate-competitive PKC inhibitor (Figure 6D). In contrast, incubation of neutrophils with the solvent DMSO did not alter parasite viability (data not shown).

Treatment of neutrophils with exogenous LTB₄ prior to infection significantly reduced the number of viable parasites (Figure 6E). To characterize the signaling pathways induced by

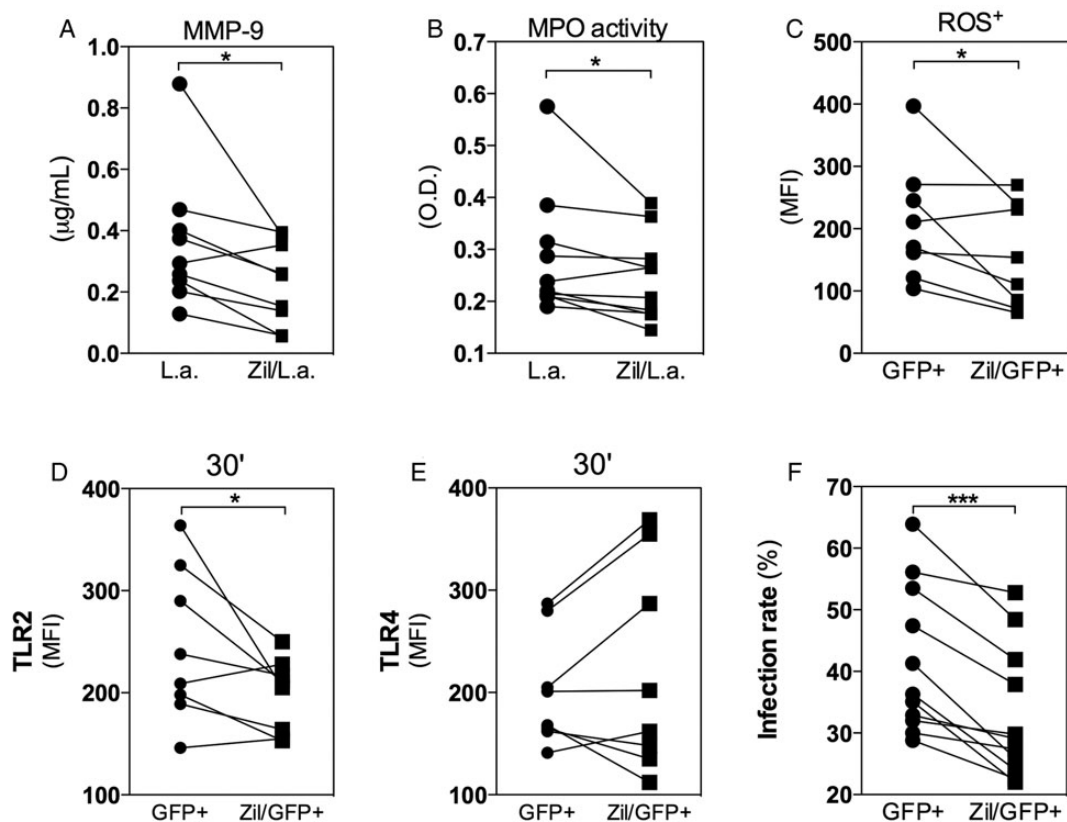


Figure 5. Degranulation and Toll-like receptor 2 (TLR2) expression in human neutrophils infected with *Leishmania amazonensis* are dependent on leukotriene B₄. Human neutrophils were treated with Zileuton for 15 minutes prior to infection with *L. amazonensis*-GFP (1 neutrophil to 10 *L. amazonensis*). After 3 hours of infection, the supernatants were collected and evaluated for degranulation by measuring metalloproteinase-9 (MMP-9) release (A) and myeloperoxidase (MPO) activity (B). Cells were harvested for flow cytometry analysis of reactive oxygen species production (C) and TLR2 (D) and TLR4 (E) expression as well as the infection rate (F). * $P \leq .05$; ** $P \leq .01$; *** $P \leq .001$.

LTB₄, we pretreated infected neutrophils with different kinase inhibitors and then added back LTB₄ prior to infection. PI3k (Figure 6F), ERK (Figure 6G), and PKC (Figure 6H) were each important for *L. amazonensis* killing, as the addition of LTB₄ did not reverse the effect observed using only the kinase inhibitors.

Inhibition of NFκB with the specific inhibitor BAY (Figure 6I) or the unspecific inhibitor Wedelolactone (data not shown) reduced LTB₄ production. However, the kinesin inhibitors LY (Figure 6J), PD (Figure 6K), and BIS (Figure 6L) did not alter LTB₄ production. These results suggest that NFκB activation plays a relevant role in lipid mediator production. Moreover, it seems that LTB₄ contributes to parasite destruction through PI3k, ERK, and PKC signaling.

Additionally, MMP-9 release was reduced following inhibition of NFκB (Figure 6M), PI3K (Figure 6N), ERK (Figure 6O), and BIS (Figure 6P). These data indicate that LTB₄ signaling can lead to degranulation mediated by PI3k, ERK, and PKC signaling, as previously demonstrated [22]. Thus, our results highlight the participation of NFκB in LTB₄ production and degranulation, which are both crucial for *L. amazonensis* control.

DISCUSSION

This study reveals the essential role played by neutrophil-derived LTB₄ in the control of *L. amazonensis* infection. In particular, the production of LTB₄ by infected neutrophils and its paracrine/autocrine effects mediate degranulation and parasite killing through the PI3k, ERK, and PKC signaling pathways. In addition, *L. amazonensis* and LPG interact with TLR2, which activates NFκB signaling. This transcription factor seems to be involved in LTB₄ production and the leishmanicidal activity of neutrophils.

The interaction between *L. amazonensis* and human neutrophils induces cellular activation, characterized by an increase in Mac-1 and a decrease in CD62L and CD16 expression at the cell surface. Our results are similar to data previously published in the literature. Resting neutrophils obtained from the peripheral blood of healthy donors were shown to express high levels of Mac-1, which was further upregulated upon stimulation [25]. In addition, ROS production also indicates the activated phenotype of infected cells, and it has been shown that ROS are highly

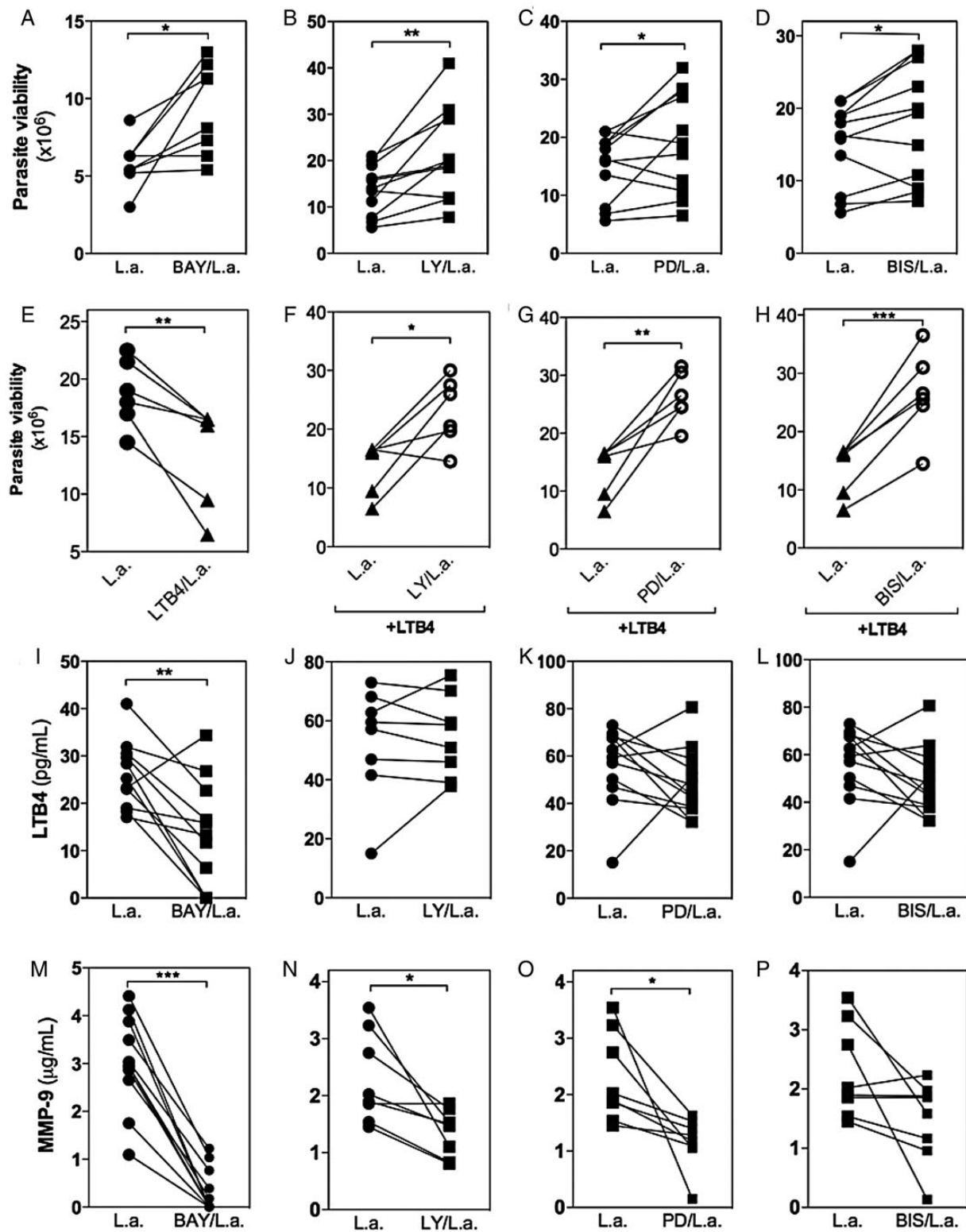


Figure 6. Signaling pathways involved in *Leishmania amazonensis* killing, leukotriene B₄ (LTB₄) production, and degranulation in human neutrophils. Neutrophils were treated or not with 3-[(4-methylphenyl)sulfonyl]-(2E)-propenenitrile (BAY) (10 μM), LY294002 (LY; 10 μM), PD98059 (PD; 50 μM), or bisindolylmaleimide (BIS; 20 nM) for 15 minutes prior to infection with *L. amazonensis*-GFP (1 neutrophil to 10 *L. amazonensis*). In some conditions, exogenous LTB₄ (10 pg/mL) was added back in the culture (F-H). After 3 hours of infection, the cells were washed, fed with supplemented Schneider's medium, and cultured at 23°C for an additional 24 hours. Parasite viability was measured by assessing the number of extracellular motile promastigotes produced (A-H). LTB₄ production (I-L) and metalloprotease-9 (MMP-9) release (M-P) were measured by enzyme-linked immunosorbent assay in the culture supernatants. **P* ≤ .05; ***P* ≤ .01; ****P* ≤ .001.

toxic and may be responsible for parasite destruction [26]. The activation of human neutrophils by LPS induced similar alterations in surface markers and ROS production [27].

Leishmania amazonensis-infected neutrophils produced LTB₄, which led to degranulation, MMP-9 release, and MPO activity. It was previously reported that LTB₄ can trigger degranulation [28] and the killing of microorganisms [10, 29, 30]. In fact, treatment of infected neutrophils with Zileuton led to an increase in *L. amazonensis* viability and a decrease in degranulation. The inhibition of parasite killing was confirmed by the ultrastructure of Zileuton-treated *L. amazonensis*-infected neutrophils, showing the crucial role of LTB₄ in the control of infection.

The interaction between *Leishmania* LPG and host cells has been extensively described [31]. We observed that LPG also induced LTB₄ production and degranulation by human neutrophils. Lipoproteins and peptidoglycans, including LPG, are recognized by TLR2 [32]. Our results showed that *L. amazonensis*-infected neutrophils presented an increase in TLR2 expression at an early time post-infection, whereas after 3 hours, there was a significant reduction. Although the role of TLRs in the engulfment of pathogens remains controversial, it has been reported that TLR2 enhances phagocytosis of *Listeria monocytogenes* by macrophages [33] and plays an important role in phagocytosis of *Streptococcus pneumoniae* by mouse neutrophils [34]. Furthermore, it was recently demonstrated that TLR2 could mediate the phagocytosis of *Staphylococcus aureus* in a mouse macrophage cell line through the MAPK signaling pathway [35]. Although the infection rate and number of internalized parasites were reduced by anti-TLR2-neutralizing antibody, based on our results, we cannot unequivocally state that TLR2 is a phagocytic receptor for *Leishmania*. Moreover, signaling through TLRs activates different pathways that mediate phagocytosis [24]. In this way, the conclusion from our findings is that TLR2 could participate in *Leishmania* internalization through PI3k and/or MAPK activation or be internalized with other phagocytic receptors. Nevertheless, we cannot exclude the possibility that during *Leishmania* phagocytosis by neutrophils, TLR2 may be included in a lipid raft that contains different phagocytic receptors already described for *Leishmania* [36]. Future studies should be conducted to address this issue.

Previous studies have shown an association between TLR signaling and lipid mediators [16, 37]. Our results further indicate that this interaction may lead to NFκB activation, possibly through TLR2, and LTB₄ production. The mechanisms involved in the production of eicosanoids following TLR ligand stimulation remain incompletely defined and may involve various processes. LPS increases arachidonic acid availability and induces the expression of enzymes responsible for eicosanoid production [38, 39]. Similarly, a β-glucan from the *Histoplasma capsulatum* cell wall induces lipid body formation through TLR2 [40]. Based on our findings, we suggest the existence of a positive loop between NFκB and 5-LO. In particular, TLR2

signaling culminates in NFκB activation, which induces expression of the enzymes responsible for eicosanoid production, such as 5-LO. Therefore, this increased production of 5-LO may produce higher levels of LTB₄ that, via a paracrine/autocrine mechanism, may amplify NFκB activation.

Furthermore, the synthesis of LTB₄ is necessary for optimal MyD88 expression and NFκB activation in mouse macrophages [41]. Additionally, treatment of human neutrophils with LTB₄ in vitro potentiates the TLR response [42]. Similar results were also obtained for the pattern recognition receptor dectin-1, a C-type lectin receptor that recognizes β-glucans [43] and whose expression is controlled by LTB₄ in mouse macrophages [44].

LTB₄ is the agonist for 2 membrane G protein-coupled receptors, BLT1 and BLT2 [45]. Our use of a BLT1 antagonist confirmed its role in parasite killing by human neutrophils. Similar results were obtained with mouse macrophages infected with *L. amazonensis* and treated with antagonists for BLT1 receptor [10]. However, an antagonist for the cysteinyl leukotriene receptors (CysTL1 and CysTL2) demonstrated no effect on the leishmanicidal activity of macrophages [10].

The role of PI3K, MEK, and PKC signaling in neutrophil degranulation and LTB₄ production during *L. amazonensis* infection was demonstrated in the current study. Degranulation by human neutrophils and eosinophils was previously shown to be PI3k dependent [46]. Our results showed that LTB₄ production was not affected by treatment with kinase inhibitors. However, a decrease in MMP-9 release and MPO activity was observed along with an increase in parasite viability. Together, these data imply a role for these enzymes downstream of LTB₄ production. These factors may also be necessary for neutrophil degranulation, as previously shown [47]. The role of LTB₄ as an activator of MAPK family members has been established [48], and PKC is directly involved in multiple steps of TLR signaling pathways [49].

In this study, we uncovered a novel role for neutrophil-derived LTB₄. Our results indicate that *L. amazonensis* may be recognized by TLR2, which stimulates neutrophil activation. This activation then leads to neutrophil degranulation via a LTB₄-dependent autocrine/paracrine mechanism. NFκB activation is also involved in LTB₄ production, and its effects, via BLT1 receptor, contribute to parasite killing. Furthermore, these inflammatory mechanisms may also be responsible for the resolution of other infections.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org/>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

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