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Leukotrienes Are Essential for the Control of *Leishmania amazonensis* Infection and Contribute to Strain Variation in Susceptibility[1]

Carlos H. Serezani,[2]*† Joao H. Perrela,* Momtchilo Russo,* Marc Peters-Golden,† and Sonia Jancar*

Leukotrienes (LTs) are known to be produced by macrophages when challenged with *Leishmania*, but it is not known whether these lipid mediators play a role in host defense against this important protozoan parasite. In this study, we investigated the involvement of LTs in the in vitro and in vivo response to *Leishmania amazonensis* infection in susceptible (BALB/c) and resistant (C3H/HePAS) mice. Pharmacologic or genetic deficiency of LTs resulted in impaired leishmanicidal activity of peritoneal macrophages in vitro. In contrast, addition of LTB_4 increased leishmanicidal activity and this effect was dependent on the BLT1 receptor. LTB_4 augmented NO production in response to *L. amazonensis* challenge, and studies with a NO synthesis inhibitor revealed that NO was critical for the enhancement of macrophage leishmanicidal activity. Interestingly, macrophages from resistant mice produced higher levels of LTB_4 upon *L. amazonensis* challenge than did those from susceptible mice. In vivo infection severity, as assessed by footpad swelling following s.c. promastigote inoculation, was increased when endogenous LT synthesis was abrogated either pharmacologically or genetically. Taken together, these results for the first time reveal an important role for LTB_4 in the protective response to *L. amazonensis*, identify relevant leishmanicidal mechanisms, and suggest that genetic variation in LTB_4 synthesis might influence resistance and susceptibility patterns to infection. ***The Journal of Immunology,*** **2006, 177: 3201–3208.**

Infection by protozoan parasites of the *Leishmania* genus represents an important public health problem in >80 countries around the world, with 2 million new cases each year (1). *Leishmania amazonensis* is a member of the *Leishmania mexicana* complex. In South American countries, it can cause a broad spectrum of clinical manifestations, ranging from single cutaneous lesions to multiple disfiguring nodules and even visceral complications. At present, the molecular and genetic basis for the development of different clinical diseases following infection with *L. amazonensis* is undefined.

Murine models of infection with the Old World species *Leishmania major* demonstrate that outcome of disease is determined by the nature (i.e., Th1 or Th2 cells) and magnitude of the T cell and cytokine responses early in infection. In infected inbred mice (such as C57BL/6 or C3H/HePas), the production of IFN-γ by Th1 cells and NK cells mediates resistance, whereas production of Th2 cell-derived cytokines confers susceptibility (2). However, outcomes in infection with the New World species *L. amazonensis* are less clearly related to Th1/Th2 polarization (3–6) This led us to speculate whether differences in the synthesis of lipid mediators involved in the early phases of infection might influence patterns of resistance and susceptibility to *L. amazonensis*.

Although best known for their participation in inflammatory diseases such as asthma (7) and atherosclerosis (8), there is increasing recognition that leukotrienes (LTs)[3] are also important in protective host responses to infection. They have been shown to be critical for the in vivo clearance of various types of microbes and in mediating the phagocytic and microbicidal capacities of phagocytes (9). LTs are derived from the metabolism of the cell membrane fatty acid arachidonic acid via the enzyme 5-lipoxygenase (5-LO), in concert with its helper protein 5-LO-activating protein (FLAP) (10). The two principal bioactive classes of LTs include LTB_4 and the cysteinyl-LTs (cysLTs), LTC_4, LTD_4, and LTE_4 (10).

LTs are also involved in the control of protozoan infections. Wirth et al. (11, 12) reported that both LTB_4 and LTC_4 increased the phagocytosis and killing of *Trypanosoma cruzi* by peritoneal macrophages. The IFN-γ-mediated killing of *Toxoplasma gondii* by human monocytes was shown to be dependent on LT biosynthesis (13). In addition, Talvani et al. (14) showed that during *T. cruzi* infection, LTB_4 is able to promote NO release and thereby kill this parasite.

It has been shown that 5-LO products are produced during in vivo and in vitro infection with *Leishmania donovani* (15, 16). However, there is no information on whether LTs participate in the host response to leishmanial infection. In this work, we sought to determine the role of specific LTs in leishmanicidal activity of macrophages in vitro, and in the control of infection in vivo, by studying both susceptible and resistant mouse strains.

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[3] Abbreviations used in this paper: LT, leukotriene; 5-LO, 5-lipoxygenase; FLAP, 5-LO-activating protein; cysLT, cysteinyl LT; iNOS, inducible NO synthase; KO, knockout; WT, wild type.

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Materials and Methods

Reagents

M199, RPMI 1640, and thioglycolate were purchased from Invitrogen Life Technologies. L-NAME (NO synthase inhibitor), L-glutamine, penicillin, streptomycin, and peroxidase-labeled monoclonal anti-rabbit IgG were all purchased from Sigma-Aldrich. LTB$_4$, U75302 (BLT1 receptor antagonist), and MK571 (cysLT1 antagonist) were purchased from BIOMOL. MK0591 (FLAP inhibitor) was donated from Merck-Frost. The rabbit antiserum to inducible NO synthase (iNOS) was from Cayman Chemical. Compounds requiring reconstitution were dissolved in either ethanol or DMSO. Required dilutions of all compounds were prepared immediately before use, and equivalent quantities of vehicle were added to the appropriate controls.

Cell viability

All compounds and vehicles used in the experiments showed no adverse effects on macrophage or *L. amazonensis* viability as determined by a cell-based MTT assay (data not shown).

Parasite

Promastigotes of *L. amazonensis* (MHOM/BR/73/M2269) were derived from amastigotes isolated from the infected footpad of BALB/c mice and resuspended in M199 plus 10% FBS for a maximum of six passages. The experiments were performed with parasites in stationary phase (5 days in culture).

Animals

Eight-week-old female 5-LO knockout (KO) (129-Alox5^{tm1Fun}) (17) and strain-matched wild-type (WT) sv/129 mice were obtained from The Jackson Laboratory and kept at our own animal facilities (Institute of Biomedical Science Animal House). Eight-week-old female BALB/c and C3H/HePas mice were bred and kept at our own facilities. Animals were kept under conventional conditions with free access to food and water. Animal protocols were approved by the University of São Paulo Committee on Use and Care of Animals.

Cell harvest

Macrophages were harvested from the peritoneal cavities of the mice by lavage with PBS 4 days after the injection of 1 ml of 3% thioglycolate as described (18). Contaminating RBC were lysed with H_2O and the cells were washed two times with PBS. The percentage of macrophages was determined microscopically using a modified Wright-Giemsa stain and a typical experiment yielded ~80% macrophages.

Macrophage leishmanicidal activity

Approximately 2–3 $\times$ 10^5 cells were allowed to attach for 60 min to round, 13-mm-diameter glass coverslips placed in 24-well plates (Costar) containing 0.5 ml of RPMI 1640. The nonadherent cells were removed by three washings in warm medium. The adherent cells were incubated in RPMI 1640 supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin (0.1 mg/ml) for 18 h at 37°C in 5% CO_2. The cells were pretreated with MK0591, U75302, MK571, or L-NAME for 30 min before addition of LTB$_4$ or LTD$_4$ in the concentrations indicated in the legends for 5 min before infection with *L. amazonensis* at a ratio of 5 promastigotes:macrophage. Preliminary dose-response experiments were conducted for each drug tested and in all cases, data are presented at the concentration which showed greatest inhibitory effect on macrophage leishmanicidal activity (data not shown). After 4 h the glass coverslips were washed three times to remove noningested parasites and 24 h after infection, the coverslips were washed with PBS, stained with HEMA 3 stain, dried, mounted on glass slides, and examined microscopically. The number of infected macrophages and the average number of parasites per macrophage were determined in 200 cells. The results were expressed as the infection index, which is the percentage of infected macrophages multiplied by the average number of amastigotes per macrophage (18).

Preparation of cell lysate

A total of 4 $\times$ 10^6 cells/well was plated in 6-well culture cell plates (Corning Costar) and stimulated with the indicated concentrations of LTB$_4$. The cells were washed twice with ice-cold PBS and then lysed by treatment for 10 min with 50 μl of ice-cold lysis buffer (10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 2% Nonidet P-40, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, and 5 μM leupeptin). The lysed cell preparation was centrifuged at 10,000 $\times$ *g* for 5 min at 4°C. Protein content in the supernatant was determined using the BCA protein assay kit (Pierce) according to the manufacturer's protocols and was adjusted to 20 μg/well.

SDS-PAGE and immunoblotting

Cell lysate was mixed with 4 μl of 5$\times$ loading buffer (50 mM Tris-HCl (pH 6.8), 2% SDS, 100 mM DTT, 10% glycerol, and 0.1% bromphenol blue). Heated samples of equal amounts of protein (20 μg/20 μl) were applied to 8% SDS-polyacrylamide gels and subjected to electrophoresis. The separated proteins were transferred to nitrocellulose membranes in Trans-blot SD-Semidry Transfer Cells (Bio-Rad; 15 min at 15 mV). After transfer, the membranes were incubated in TBST buffer (150 mM NaCl, 20 mM Tris, 0.01% Tween 20 (pH 7.4)) containing 5% fat-free dry milk. The blot was treated with a 1/1000 dilution of rabbit polyclonal Ab to iNOS for 1 h at room temperature, then washed three times with TBST, and incubated with 1/5000 dilutions of peroxidase-conjugated monoclonal anti-rabbit IgG for 1 h at room temperature. The immunocomplexed peroxidase-labeled Abs were visualized by an ECL chemiluminescence kit following the manufacturer's instruction (Amersham Biosciences).

Measurement of nitrite levels

To evaluate NO production, nitrite concentration in the supernatants of macrophage cultures was measured using the standard Griess reaction (18). Briefly, 50 μl of the culture supernatant was reacted with 50 μl of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, and 2.5% H_3PO_4) for 10 min at room temperature. The absorbance was measured at 540 nm by using a 620-nm reference filter in a Dynatech microplate reader and the nitrite concentration was calculated by using a standard curve of sodium nitrite. All tests were done at least in triplicate.

Measurement of LTs

Macrophages from BALB/c or C3H/HePas mice (2 $\times$ 10^5 cells/well) were cultured in 96-well plates in RPMI 1640. Cultures were then incubated for 2, 4, 8, and 24 h at a ratio of 5:1 *L. amazonensis*: macrophage. Supernatants were collected and LTB$_4$ and cysLT levels were quantified by enzyme immunoassay according to the manufacturer (Cayman Chemical). The limits of assay detection for LTB$_4$ and cysLTs are 3.9 and 7.8 pg/ml, respectively.

In vivo treatment and infection

The mice were treated with 1 mg/kg zileuton i.p. 1 h before infection and once daily for 7 days thereafter. A total of 1 $\times$ 10^6 stationary phase promastigotes of *L. amazonensis* was inoculated s.c. into the left hind footpad of 8-wk-old BALB/c, C3H/HePas, 5-LO KO, and the counterpart WT female mice (at least five mice per group). The evolution of the disease was monitored biweekly over the next 10 wk by measuring footpad thickness with a paquimeter (Mitutoyo). Results are expressed as the difference in thickness between the infected and the noninfected contralateral footpad.

Statistical analysis

Data are represented as mean $\pm$ SEM and were analyzed with the Prism 3.0 statistical program (GraphPad Software). Comparisons between two experimental groups were performed using Student's *t* test. Comparisons among more than or equal to three experimental groups were performed by ANOVA followed by the Bonferroni test. Differences were considered significant if $p \leq 0.05$. All experiments were performed on more than or equal to three separate occasions unless otherwise specified.

Results

5-LO metabolites increase macrophage leishmanicidal activity

Infection index of macrophages from susceptible BALB/c mice was generally higher than that from resistant C3H/HePas mice, as expected. Under all conditions, the infection index was higher at 24 than at 4 h of incubation (data not shown). Changes in infection index observed with experimental treatments were qualitatively similar at both time points, but only 24 h data will be presented.

Pharmacologic inhibition of LT synthesis with the FLAP inhibitor MK0591 increased the infection index of macrophages from both susceptible and resistant mice (Fig. 1, *A* and *B*). Although these results were obtained with thioglycolate-elicited macrophages, a similar decrease in leishmanicidal activity was also observed with MK0591 treatment of resident peritoneal macrophages from BALB/c mice (data not shown). We verified the importance

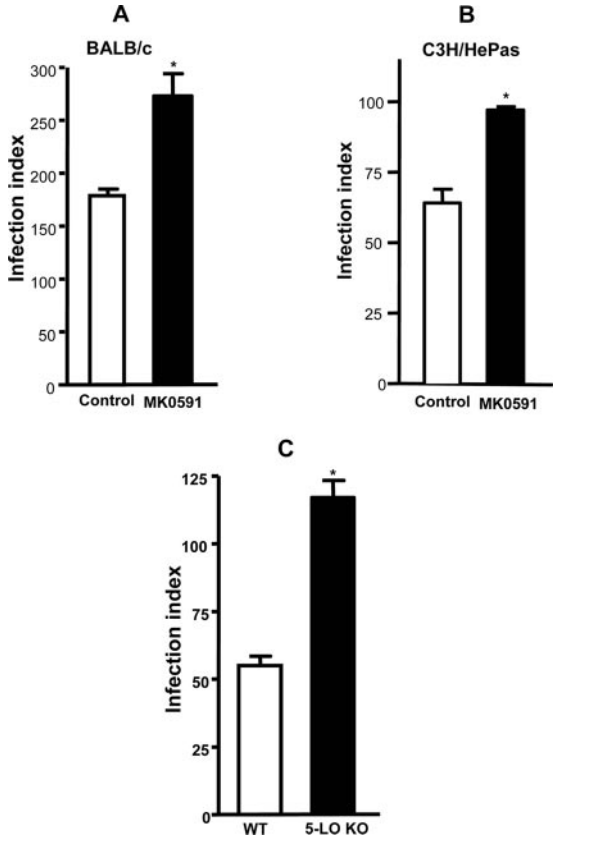

FIGURE 1. Endogenous LTs increase leishmanicidal activity of macrophages. Macrophages from BALB/c (*A*), C3H/HePas (*B*), and 129 5-LO KO or WT (*C*) mice were infected with the promastigote form of *L. amazonensis* after 30 min pretreatment with or without the FLAP inhibitor MK0591 (1 μM). After 24 h, the infection index was determined as described in *Materials and Methods.* Data are expressed as the mean ± SE of triplicate values from one experiment representative of a total of three. *, $p < 0.05$ vs control or WT.

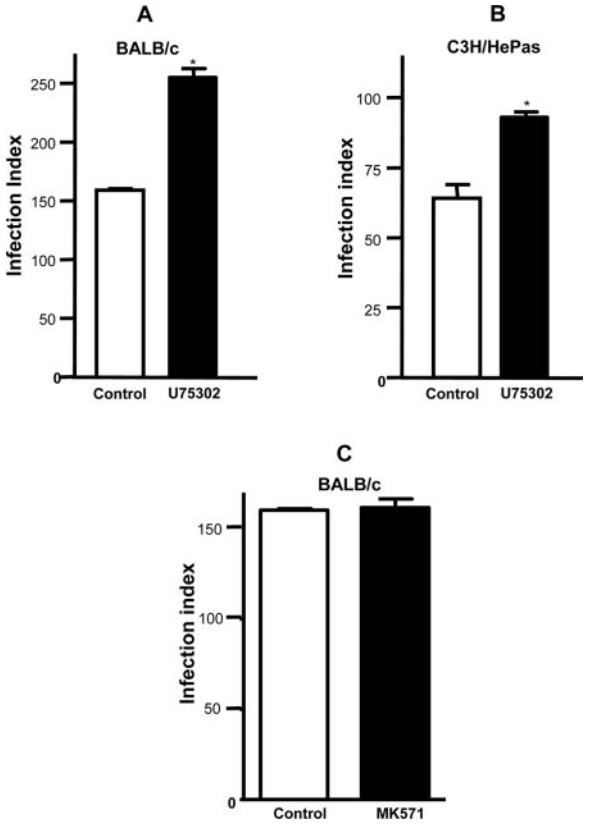

FIGURE 2. Role of specific LT receptors in the leishmanicidal activity of macrophages. Macrophages from BALB/c (*A* and *C*) or C3H/HePas (*B*) mice were infected with the promastigote form of *L. amazonensis* after 30 min pretreatment with or without the BLT1 antagonist U75302 (1 μM) (*A* and *B*) or the cysLT1 antagonist MK571 (10 μM) (*C*). After 24 h, the infection index was determined. Data are expressed as the mean ± SE of one experiment representative of a total of three. *, $p < 0.05$ vs control.

of endogenous LTs in leishmanicidal activity by using macrophages from 5-LO-deficient mice. As can be observed in Fig. 1*C*, macrophages from 5-LO KO mice showed impaired leishmanicidal activity (~112% increase in the infection index) when compared with macrophages from WT mice. These results suggest that LTs produced by macrophages following infection with *L. amazonensis* promastigotes support their capacity to kill the parasite. We have also observed the same effects of LT biosynthesis inhibition in macrophage infection by the Old World parasite *L. major* (data not shown).

To investigate which LTs are responsible for the increased leishmanicidal activity, we pretreated macrophages with the BLT1 antagonist U75302 or the cysLT1 antagonist MK571. Antagonism of BLT1 in macrophages from both BALB/c (Fig. 2*A*) and C3H/HePas (Fig. 2*B*) mice increased the infection index when compared with the untreated control. However, antagonism of cysLT1 with MK571 had no effect on BALB/c macrophage infection (Fig. 2*C*).

Because these data suggested a role for endogenous LTB_4 in macrophage leishmanicidal activity, we wished to confirm that exogenous LTB_4 was capable of directly enhancing it. Indeed, the addition of LTB_4 dose-dependently increased leishmanicidal activity in both susceptible and resistant mice. However, this effect was more pronounced in macrophages from BALB/c than from C3H/HePas mice (Fig. 3, *A* and *B*). Importantly, the large difference in infection index between macrophages from susceptible and resistant mice under control conditions was abolished by 100 nM LTB_4 treatment of cells from susceptible animals. Although our data showed no effect of endogenous cysLTs on the infection index, we wished to determine whether the BALB/c macrophages were able to respond to exogenous cysLTs. As can be observed in Fig. 3*C*, the addition of 100 nM LTD_4 enhanced macrophage leishmanicidal activity when compared with untreated control, though not to the same degree as did LTB_4 (Fig. 3*A*).

Macrophage LT synthesis in response to L. amazonensis *infection*

We next sought to verify that LTB_4 was indeed generated upon macrophage challenge with promastigotes in vitro, and compare the responses of cells from susceptible and resistant strains. Fig. 4 shows the time course of LTB_4 production by infected macrophages. Significant increases in LTB_4 production over the uninfected control level (measured at 24 h) were observed by 2 h in both strains. C3H/HePas macrophages produced significantly higher levels of LTB_4 than BALB/c cells at all time points tested. After a plateau in synthesis reached at 8 h in both strains, a further increment in LTB_4 accumulation at 24 h was noted only in the C3H/HePas cells (Fig. 4). The levels of cysLTs in macrophage culture supernatant were below the detection limit of the assay (7.9 pg/ml) at all time points tested (data not shown). This result is in accordance with data in Fig. 2*C* showing that the cysLT1 antagonist had no effect on macrophage leishmanicidal activity.

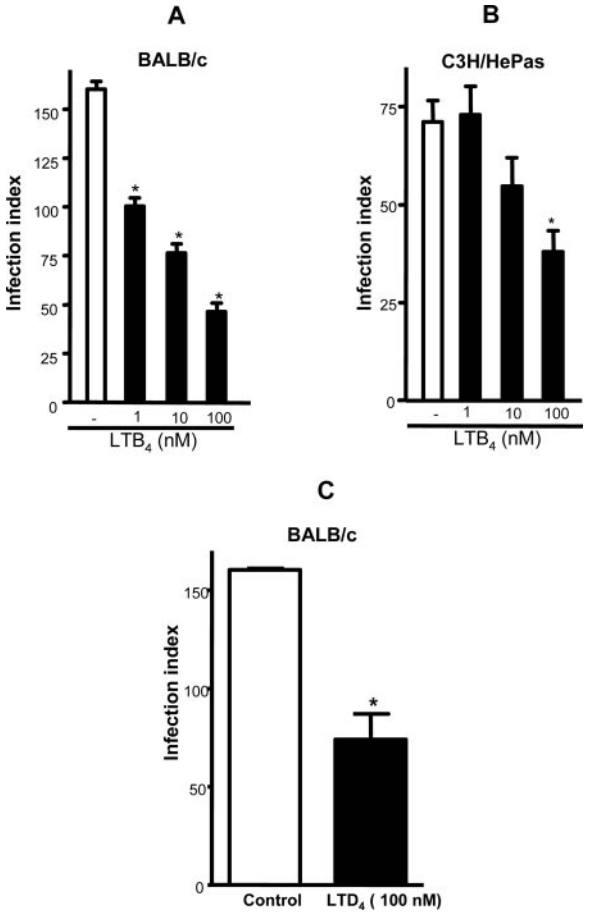

FIGURE 3. Exogenous LTB$_4$ increases leishmanicidal activity of macrophages. Macrophages from BALB/c (*A* and *C*) or C3H/HePas (*B*) mice were infected with the promastigote form of *L. amazonensis* after 5 min pretreatment with or without the indicated concentrations of LTB$_4$ (*A* and *B*) or 100 nM LTD$_4$ (*C*). After 24 h, the infection index was determined. Data are expressed as the mean ± SE of one experiment representative of a total of three. ∗, $p < 0.05$ vs control.

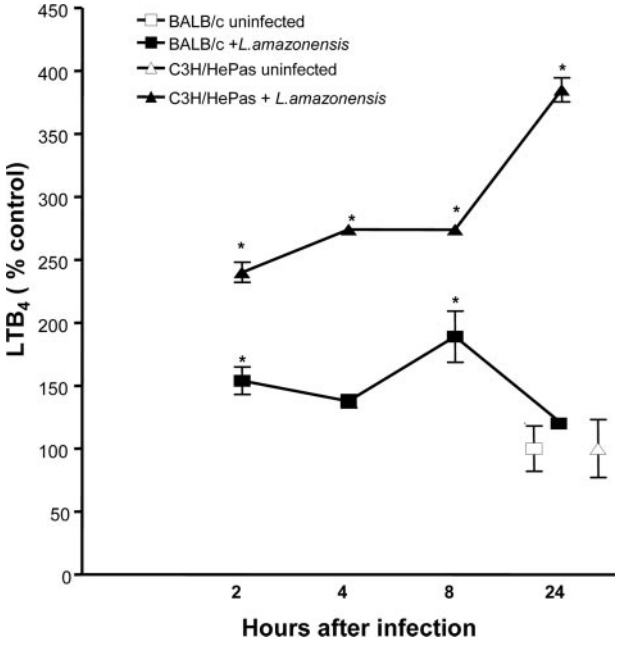

FIGURE 4. LTB$_4$ production in macrophages from BALB/c and C3H/HePas mice infected with *L. amazonensis*. LTB$_4$ levels were measured as described in *Materials and Methods* in BALB/c or C3H/HePas macrophage supernatants at different time points following infection with the promastigote form of *L. amazonensis*. Data are expressed as the mean ± SE from two independent experiments, each performed in triplicate. ∗, $p < 0.05$ vs the uninfected control by ANOVA. LTB$_4$ level of uninfected control supernatant were 2.73 ± 0.63 (C3H/HePas) and 3.20 ± 0.94 (BALB/c) after 24 h of culture.

LTB$_4$ enhances in vitro leishmanicidal activity through the induction of NO formation

NO is well-established as a mediator involved in the control of *Leishmania* infection. The importance of NO in the control of *L. amazonensis* infection in vitro was verified by the fact that treatment with the NO synthesis inhibitor L-NAME (1 mM) enhanced the infection index of BALB/c macrophages. We next wished to determine whether NO was the microbicidal molecule responsible for the ability of LTB$_4$ to enhance killing. BALB/c macrophages incubated with or without LTB$_4$ (100 nM) were pretreated or not with L-NAME 30 min before infection. The ability of exogenous LTB$_4$ to enhance leishmanicidal activity was abolished by the inhibitor of NO synthesis (Fig. 5).

We further evaluated the ability of exogenous LTB$_4$ to induce NO secretion and iNOS expression. As can be observed in Fig. 6, *A* and *B*, macrophages from both strains induced significant levels of nitrite (a final product derived from NO production) after treatment with 100 nM LTB$_4$, when compared with untreated macrophages. Of note, the cells from BALB/c mice manifested a greater response and did so at a lower LTB$_4$ concentration as compared with cells from C3H/HePas mice. In addition, iNOS expression in macrophages from BALB/c mice also increased dose-dependently after LTB$_4$ stimulation (Fig. 6*C*).

We also evaluated the effect of LTB$_4$ on levels of nitrite generated in *L. amazonensis*-infected macrophages. BALB/c macrophages were pretreated with different doses of LTB$_4$ for 5 min

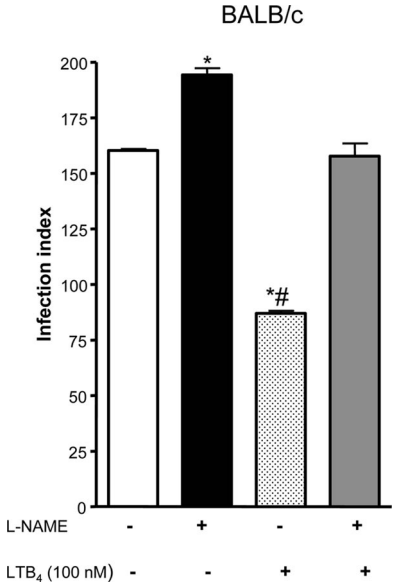

FIGURE 5. LTB$_4$-induced leishmanicidal activity is dependent on NO production. Macrophages from BALB/c were infected with the promastigote form of *L. amazonensis* after 30 min pretreatment with or without the NO synthase inhibitor L-NAME (1 mM) or LTB$_4$ (100 nM). After 24 h, the infection index was determined. Data are expressed as the mean ± SE of one experiment representative of a total of three. ∗, $p < 0.05$ vs control; #, $p < 0.05$ vs L-NAME by ANOVA.

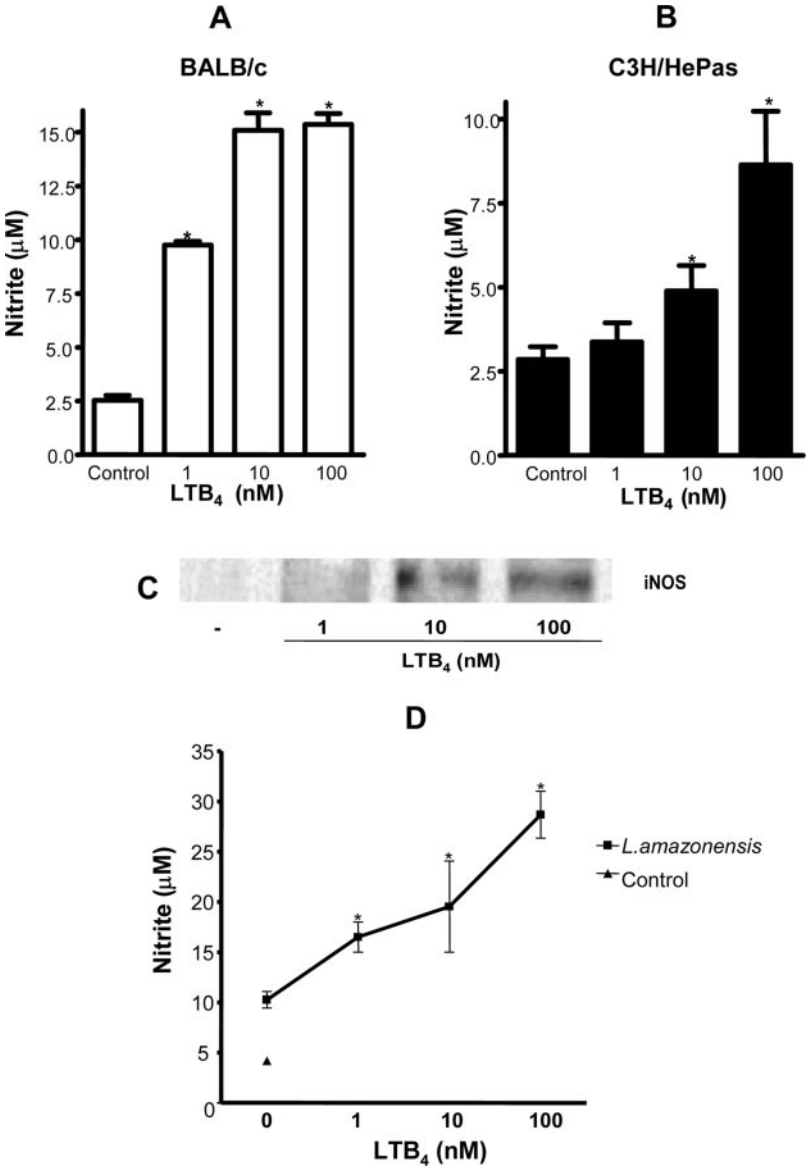

FIGURE 6. Effect of LTB_4 on NO formation in murine macrophages. Macrophages from BALB/c (*A*) or C3H/HePAS (*B*) mice were stimulated with the indicated concentrations of LTB_4 and after 24 h, the supernatants were collected and the nitrite concentration was determined by the Griess reaction. Data are expressed as the mean ± SE from triplicate values from one experiment representative of a total of three. *, $p < 0.05$ vs control. *C*, Macrophages from BALB/c mice were plated (4×10^6 cells/well) and incubated in the presence or absence of different concentration of LTB_4. After 24 h, the samples of lysed cells (20 μg of protein) were subjected to Western blot analysis. Results from one experiment of two are shown. *D*, Macrophages from BALB/c were treated with different LTB_4 concentrations for 5 min followed by *L. amazonensis* infection. After 24 h, the supernatants were collected and the nitrite concentration was determined by Griess reaction. Data are expressed as the mean ± SE of triplicate values from one experiment representative of a total of three. *, $p < 0.05$ vs infected in the absence of LTB_4 stimulation.

before the infection. While *L. amazonensis* infection induced NO release, treatment with LTB_4 dose-dependently increased NO levels (Fig. 6*D*).

LTs modulate the in vivo L. amazonensis *infection*

The in vivo infection of susceptible BALB/c mice with *L. amazonensis* promastigotes resulted in a greater degree of footpad swelling after 10 wk than in the resistant C3H/HePas mice. Of note, the degree of footpad swelling in the WT sv/129 animals was less than that of other mouse strains at all time points observed. To verify the importance of LTs in the control of *L. amazonensis* infection in vivo, we used pharmacological and genetic approaches. First, BALB/c or C3H/HePas mice were treated daily with the LT synthesis inhibitor zileuton (1 mg/kg) for the first 7 days following inoculation in the left hind footpad with 1×10^6 promastigotes. The footpad swelling was measured every 2 wk. Both mouse strains exhibited an increase in lesional size with zileuton treatment which was apparent at 4 wk and maximal at 8 wk postinfection (Fig. 7, *A* and *B*). The time course curves for zileuton were left shifted as compared with those for vehicle. By 10 wk of infection, the lesion in zileuton-treated C3H/HePas mice was no longer different from that in untreated mice (Fig. 7*B*). Mice genetically deficient in the *5-LOX* gene and thereby unable to synthesize LTs also exhibited increased lesional size at weeks 4–10 (Fig. 7*C*).

Discussion

This study for the first time establishes the importance of LTs in both in vivo and in vitro leishmanicidal activity and identifies LTB_4 as the macrophage-derived species involved in this phenomenon. The relevant findings are: 1) endogenous and exogenous LTs increase in vitro leishmanicidal activity of macrophages obtained from susceptible and resistant mice; 2) LTB_4 appears to be the major molecule that mediates resistance and its effects are exerted via the BLT1 receptor; 3) after *L. amazonensis* infection, macrophages from resistant mice produce higher levels of LTB_4 when compared with macrophages from susceptible mice; 4) the enhanced leishmanicidal activity induced by LTs is dependent on NO production; 5) LTs are relevant mediators for the in vivo control of *L. amazonensis* infection in both susceptible and resistant mice.

It has been demonstrated in different models of infection that LTs increase phagocyte effector functions, including phagocytosis, microbicidal activity, generation of reactive oxygen and nitrogen species, and a myriad of proinflammatory cytokines (9). Our group

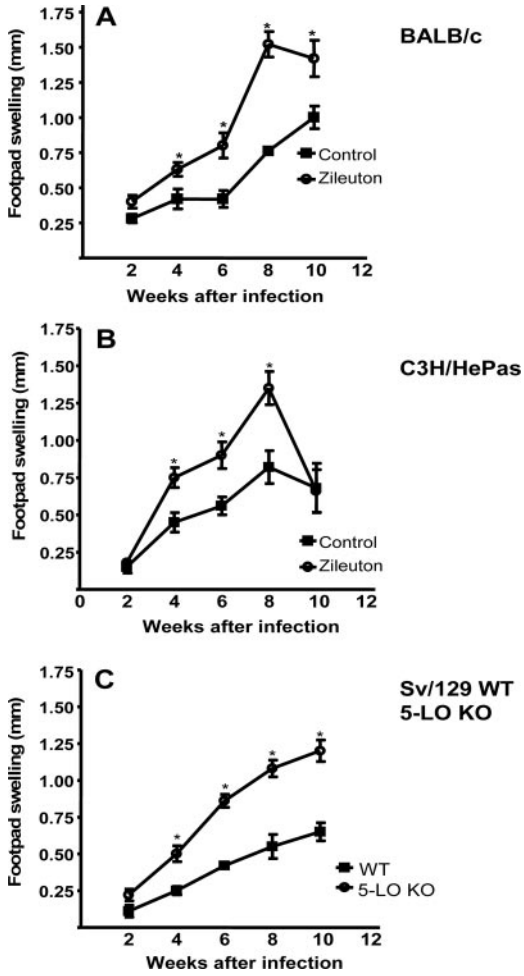

FIGURE 7. Role of LTs in the in vivo response to *L. amazonensis* infection in resistant and susceptible mice. BALB/c (*A*) or C3H/HePas (*B*) mice were inoculated with 10^6 stationary phase promastigotes of *L. amazonensis* in the left hind footpad at time 0, and were treated or not from days 1–7 with the 5-LO inhibitor zileuton (1 mg/kg i.p. daily). Each group consisted of five to seven mice. 5-LO KO and their strain-matched WT mice (*C*) were also infected as described above. The course of infection was monitored biweekly by measuring the increase in footpad thickness with a paquimeter, which is expressed as the degree of swelling after subtraction of the thickness of the contralateral uninfected footpad.

has demonstrated that LTs increase phagocytosis of IgG- and complement-opsonized targets as well as microbicidal activity, and have identified a number of the relevant signal transduction events which are amplified (19–22). Because infection with the promastigote form of the *Leishmania* parasite does not require opsonization, we sought to determine the importance of LTs in a model of protozoan infection. Thus, we evaluated whether LTs would influence the outcome of *L. amazonensis* infection in vitro and in vivo.

The immune mechanisms that underpin resistance/susceptibility to *Leishmania* infections and particularly to *L. amazonensis* infection are as yet uncertain. It is becoming increasingly apparent that the nature of the immune response which dictates outcomes are variable and dependent on both the mouse strain and the *Leishmania* species (3, 4, 23, 24).

With respect to *L. amazonensis* infection, it is now clear that BALB/c mice develop tumor-like lesions, followed by the dissemination of the parasite, while the CH3/HePas animals develop a small local lesion which resolves spontaneously (2, 5, 25–28). With this in mind, we asked whether LTs might influence the susceptibility phenotype. Both pharmacological and genetic approaches indicated that LTs are important mediators in the control of leishmanicidal activity in macrophages from both susceptible BALB/c and resistant C3H/HePas mice. We also performed experiments in macrophages from WT sv/129 or 5-LO KO mice. There are no reports in the literature regarding infection of *L. amazonensis* in sv/129 mice. We found that this strain resembles the resistant C3H/HePas mice. Indeed, WT sv/129 did not exhibit any increase in the infection index between 4 and 24 h of infection (data not shown). However, macrophages from 5-LO-deficient mice were unable to control leishmanial infection at 24 h of infection. Our in vitro experiments indicated that LTB$_4$ is the major LT involved in the leishmanicidal activity of macrophage, because unlike cysLTs, it was produced and antagonism of its high-affinity BLT1 receptor increased infection index. These results are in line with our previous work showing that LTB$_4$ was the major LT involved in the bactericidal activity of alveolar macrophages (22). It has been demonstrated that exogenous LTB$_4$ and LTC$_4$ enhanced phagocytosis and killing of *T. cruzi* (11, 12). This is in accordance with our findings that exogenous LTD$_4$ was able to enhance macrophage leishmanicidal activity. In our model, LTs promotes killing but did not influence the uptake of unopsonized *L. amazonensis* (data not shown). Promastigotes can attach to the macrophage via the mannose-fucose receptor, which binds to mannan residues of the lipophosphoglycan in the promastigote forms (29–31). It is not known whether LTs can modulate the signal through the mannose receptor.

The mechanisms that underlie resistance and susceptibility to *L. amazonensis* infection are still elusive. Differences in the generation of IL-10 (32, 33), TFG-β (34), and NO (35), and in the response to IL-12 (5) and IFN-γ (36, 37), have all been suggested. However, no previous reports have considered the role of lipid mediators in the resistance and susceptibility to infection. Kuroda et al. (38) showed that BALB/C c mice were more sensitive to the suppressive effect of PGE$_2$ as compared with C3H/HePas and C57BL/6 mice and this effect was due to a higher number of PGE$_2$-binding sites than those of other mouse strains. The fact that BALB/c macrophages tended to exhibit a greater increase in the leishmanicidal activity (Fig. 3) and NO generation (Fig. 6) in response to lower concentrations of LTB$_4$ might be consistent with a similar difference in BLT1 expression. However, we found that C3H/HePas macrophages produced 3-fold more LTB$_4$ than BALB/c macrophages when challenged with *L. amazonensis*. The levels of LTB$_4$ found were rather low and this might in part be explained by the well-known attenuated eicosanoid synthetic capacity of thioglycolate-elicited macrophages (39, 40). Steil et al. (41) previously demonstrated that immune complex-induced peritonitis was associated with greater macrophage generation of LTB$_4$ in the peritoneal cavity of C3H/HePas mice than BALB/c mice. This suggests that the higher capacity for LTB$_4$ production of C3H/HePas is not specific for *L. amazonensis* infection, but it extends to other stimuli. The mechanisms responsible for the differences in LTB$_4$ production among different strains are currently under investigation.

It is well-established that NO is involved in the control of *L. major* and *L. donovani* infection. However, it has been reported that NO inhibition did not modify the course of *L. amazonensis* infection in vitro (42) or in vivo (24). Our results with L-NAME implicated NO as a major mediator of leishmanicidal activity of LTB$_4$. However, some persistent leishmanicidal activities of LTB$_4$-treated macrophages even in the presence of L-NAME suggest that other mechanisms independent of NO may be operative. Talvani et al. (14) demonstrated that NO is the molecule involved

in LTB_4-mediated *T. cruzi* killing. However, the authors did not evaluate the relative importance of LTB_4 on NO production. We showed an enhanced production of NO in LTB_4-treated macrophages that were infected with *Leishmania*. Moreover, treatment of macrophages with LTB_4 induces iNOS expression and NO production in both strains of mice. Our results are in line with the findings of Talvani et al. (14) that showed a synergism between infection with *T. cruzi* and treatment with LTB_4.

Our findings in vivo confirmed the involvement of LTs in the control of *L. amazonensis* infection because treatment of mice with zileuton increased the footpad swelling of resistant and susceptible mice when compared with untreated control animals. This is the first report showing in vivo and in vitro *L. amazonensis* infection of sv129 mice. We found that the outcome of infection in this strain is similar to the resistant C3H/HePas strain. Interestingly, the sv/129 strain is also resistant to *L. major* infection (43–45). The importance of endogenous LTs in the in vivo control of infection has been demonstrated in different models of infection in vivo. Our group was the first to show that 5-LO-deficient mice are unable to control *Klebsiella pneumoniae* infection (46). In another model of protozoan infection, the treatment of BALB/c mice with a BLT1 antagonist increased *T. cruzi* parasitemia but not lethality (14). In our model, both pharmacological inhibition and genetic deficiency in LT biosynthesis increased the footpad swelling after *L. amazonensis* infection.

Deficiency of LT synthesis has been described in malnutrition (47, 48) and HIV infection (49–51). Those conditions are also known to predispose to reactivation of latent leishmaniasis (1, 52–54). Thus, LTs could be relevant mediators involved in the control of *Leishmania* infection in immunosuppressed patients and could be clinically important as targets for immunomodulatory therapy.

In summary, our results shows that LTB_4 plays a role in the in vivo and in vitro control of *L. amazonensis* in both susceptible and resistant mouse strains and its effect is mediated by the increase of iNOS expression and NO generation. In addition, we also observed an increase in LTB_4 generation by macrophages of resistant mice when compared with cells from a more susceptible strain. Our data implicate LTB_4 as a mediator involved in the pattern of resistance/susceptibility to infection with *Leishmania*.

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Disclosures

The authors have no financial conflict of interest.

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