Brief Definitive Report

The Inhibition of Arginase by N^{ω}-Hydroxy-L-Arginine Controls the Growth of *Leishmania* Inside Macrophages

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Abstract

Polyamine synthesis from L-ornithine is essential for *Leishmania* growth. We have investigated the dependence of *Leishmania* infection on arginase, which generates L-ornithine, in macrophages from BALB/c, C57BL/6, and nitric oxide synthase II (NOS II)-deficient mouse strains. We have found that N^{ω}-hydroxy-L-arginine (LOHA), a physiological inhibitor of arginase, controls cellular infection and also specifically inhibits arginase activity from *Leishmania major* and *Leishmania infantum* parasites. The effect was proportional to the course of infection, concentration dependent up to 100 μM, and achieved without an increase in nitrite levels of culture supernatants. Moreover, when the L-arginine metabolism of macrophages is diverted towards ornithine generation by interleukin 4–induced arginase I, parasite growth is promoted. This effect can be reversed by LOHA. Inhibition of NOS II by N^{G}-methyl-L-arginine (LNMMA) restores the killing obtained in the presence of interferon (IFN)-γ plus lipolysaccharide (LPS), whereas the nitric oxide scavenger 2-(4-carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-3-oxide-1-oxyl (carboxy-PTIO) was without effect. However, exogenous L-ornithine almost completely inhibits parasite killing when added in the presence of LOHA to macrophages from NOS II–deficient mice or to BALB/c-infected cells activated with IFN-γ plus LPS. These results suggest that LOHA is an effector molecule involved in the control of *Leishmania* infection. In addition, macrophage arginase I induction by T helper cell type 2 cytokines could be a mechanism used by parasites to spread inside the host.

Key words: arginase • NOS II • infection • nitric oxide • L-ornithine

Introduction

Leishmania infection is one the best elucidated models for studying defense mechanisms in murine macrophages. Resistance or susceptibility to the infection in vivo is regulated by the Th1 or Th2 type of immune response (1). The inhibition of IL-12 synthesis and the induction of IL-10 and TGF-β by infected cells rank among the most important strategies used by the parasite to escape the immune control (2). However, macrophages under appropriate conditions can control infection of *Leishmania*, and this effector mechanism is triggered when cells are activated by cytokines released mainly by Th1-type $CD4^{+}$ cells. These include IFN-γ and TNF-α as inducers of nitric oxide synthase II (NOS II), the main pathway responsible for the killing of *Leishmania*. In the mouse model, parasite killing is generally accepted to occur by the toxic capacities of nitric oxide (NO), one of the products of this enzyme (3).

Activated murine macrophages and dendritic cells metabolize arginine by two alternative pathways involving either the enzymes NOS II or arginase (4). NOS II catalyzes the conversion of L-arginine into NO and citrulline in two steps. In the first one, arginine is hydroxylated to N^{ω}-hydroxy-L-arginine (LOHA) and, in the second one, LOHA is further oxidized to citrulline and NO (5). Arginase hydrolyzes arginine to ornithine and urea. In previous studies we demonstrated that the balance between the two enzymes is competitively regulated by Th1 and Th2 cells via their secreted cytokines: Th1 cells induce NOS II, whereas Th2 cells induce arginase I, one of the two enzyme isoforms. Moreover, induction of any of these enzymes is accompanied by suppression of the other, indicative of two competitive states in murine macrophages (6, 7). This competition also occurs at other levels; arginase reduces the synthesis of NO by substrate depletion (7, 8), and

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LOHA is the most effective physiological inhibitor of arginases (9). However, this oxidized amino acid is not merely an unstable intermediate in the synthesis of NO. Several studies have demonstrated that it can be secreted by cells and is present in human and rat plasma (10). Also, it has been reported that LOHA is a target of several inorganic radicals, such as superoxide (11), as well as substrate of other oxidases apart from NOS II (12), yielding alternative nitrogen derivatives. Thus, only the conversion of arginine to LOHA remains an exclusive feature of NOSs.

In contrast to the well-documented role of NOS II in macrophages, only few data exist about the functions of arginase I. Nevertheless, one of the metabolic fates of L-ornithine is polyamine synthesis. In this pathway, arginine results in ornithine through the action of arginase and is further converted to putrescine by ornithine decarboxylase (ODC).

Polyamines are essential for the growth of all trypanosomatidae. Therefore, the inhibition of polyamine synthesis is an established therapeutic approach for the treatment of parasitic diseases (13) as well as cellular growth disorders (14).

The only reported study on the regulation of arginase induction in vivo in a parasitic disease demonstrates that the killing of *Trypanosoma brucei* by NOS II induction is restored by the early induction of arginase, which competes with NOS II for substrate availability (8).

In this work we have investigated the possible roles of LOHA in the interaction between macrophages and *Leishmania,* as (a) some parasite strains have arginase activity (15), (b) LOHA is a potent inhibitor of arginases, and (c) the killing capacities of NO in infected cells have usually been demonstrated by adding NOS inhibitors that abolished both NO and LOHA generation (16).

The results obtained point to a novel defense mechanism against *Leishmania* infection, mediated by LOHA, through the inhibition of arginase from parasites. Moreover, in agreement with this finding, our data also suggest that arginase I induction in macrophages is used by the parasite to spread inside the host.

Materials and Methods

Medium and Reagents. Macrophage cultures were performed in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 60 mM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO BRL). Recombinant murine IL-4 and IFN-γ were obtained from PeproTech; LPS from *Salmonella minnesota* and L-ornithine from Sigma-Aldrich; endotoxin-free superoxide dismutase (SOD) from Roche Molecular Biochemicals; 2-(4-carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-3-oxide-1-oxyl (carboxy-PTIO) from ICN Biomedicals; and N^G-methyl-L-arginine (LNMMA) and LOHA from Alexis Corp.

Animals, Parasites, and Generation of Bone Marrow–derived Macrophages. Mice of strains BALB/c and C57BL/6, 6–8 wk of age, were purchased by Janvier España, Sociedad Limitada (Spain) and tested routinely in the Parasitology Unit for murine pathogens. C57BL/6 NOS II knockout mice were from the specific pathogen-free animal facilities of the Max-Planck-Institut and were used between 6–8 wk of age.

Leishmania infantum WHO reference strain (M/CAN/ES/88/CHUMI) and *Leishmania major* WHO reference strain (M/IR/−/173) promastigotes were isolated and propagated as published elsewhere (17). Macrophages were derived from bone marrow cells as described previously (6).

Infection of Bone Marrow–derived Macrophages with Leishmania Species and Activation with Cytokines. Stationary-phase promastigotes were added to bone marrow–derived macrophages (BMMϕ) cultures (10^6 cells/ml) in 24-well plates (COSTAR) with round cover slides, at parasite to cell ratio of 3:1. The plates remained at 26°C for 2 h. Nonphagocytosed parasites were removed by washing the cells, and then cultures were treated with 20 ng/ml IL-4, 5 ng/ml IFN-γ plus 0.1 μg/ml LPS with or without 100 μM LOHA (unless indicated), 250 μM L-ornithine, and 100 μM carboxy-PTIO or 5 mM LNMMA and transferred to a CO_2 incubator at 37°C for an infection period of 48 h, except for the kinetic experiments. Finally, the round cover slides were removed from the plates, mounted, and dyed with Giemsa staining (DiffQuick; QCA). The percentage of infected cells as well as the number of amastigotes in 100 cells were counted by microscopic examination of stained preparations.

Measurement of Arginase Activity and Nitrite Concentration in Infected BMMϕ. Arginase activity was measured in macrophage lysates and in promastigote lysates, as described previously (6). Nitrites were measured in the supernatant from all infected cultures both in the presence and the absence of carboxy-PTIO, LOHA, or LNMMA by the Griess reagent, using $NaNO_2$ as standard curve.

Cellular Viability. Experiments testing macrophage viability in the presence of LOHA were made by using the 3-[-4,5-di-

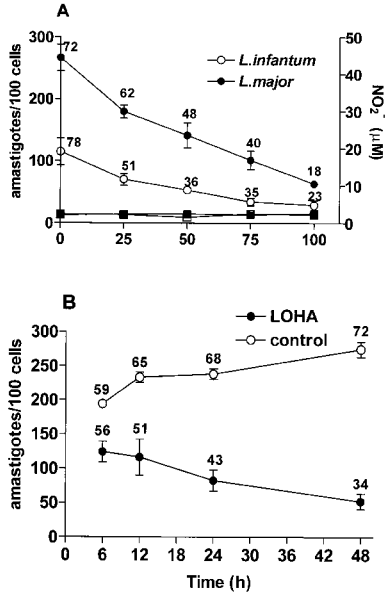

Figure 1. LOHA inhibits parasite growth in two *Leishmania* species. BMMϕ from BALB/c mice were infected with either *L. infantum* (open symbols) or *L. major* (filled symbols) promastigotes, in the presence of increasing amounts of LOHA for 48 h (A) or with 100 μM LOHA vs. time (B). Cells were used to determine both the number of intracellular parasites (circles) and the percentage of infection (numbers), whereas nitrite concentrations (squares) were measured in culture supernatants from those cells, as described in Materials and Methods.

methyltiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay, as described previously (18).

Data Presentation. Data reported are means ± SD ($n = 4$) from at least three independent experiments. Student's paired t test was used to analyze statistical significance ($P < 0.05$).

Results and Discussion

In this work we have analyzed the response of *Leishmania*-infected macrophages, under conditions in which cells are induced to have either a functional NOS II or a functional arginase I.

There are few consistent data reporting arginase activity in *Leishmania* species (15). Thus, we started measuring arginase activity in promastigote lysates of *L. major* and *L. infantum*. The specific enzyme activities, measured at 1 mM L-arginine (the concentration found in RPMI), were 11.34 ± 1.80 mU/10^7 parasites and 9.8 ± 0.5 mU/10^7 parasites, respectively. To confirm that the urea measured in the assay was derived from arginase and not from other urea-producing enzymes, we determined both activities in the presence of LOHA, the most specific arginase inhibitor commercially available (Ki ~30 μM; reference 19). We found that 100 μM LOHA decreased enzyme activity to 0.3 mU/10^7 parasites for *L. major* and 0.15 mU/10^7 parasites for *L. infantum*. This amounts to ~98% inhibition. In conclusion, these data demonstrate that both species contain a functional arginase that is efficiently inhibited by LOHA.

Analysis of LOHA-dependent Inhibition of Parasite Infection. To further assess the effect of LOHA on intracellular parasites, we infected BMMϕ with *L. major* and *L. infantum* promastigotes in the presence of increasing LOHA concentrations (Fig. 1 A) and determined the kinetics of the infection up to 48 h (Fig. 1 B). The inhibition leads to a dramatic decrease in both the number of intracellular amastigotes and the percentage of cells infected. The viability of macrophages was not compromised, as assessed by the MTT reduction assay (control: 0.75 ± 0.02 versus LOHA: 0.78 ± 0.03). Additionally, the number of remaining amastigotes was reduced by the inhibitor during the time course of infection, suggesting a cytotoxic effect of LOHA on parasites.

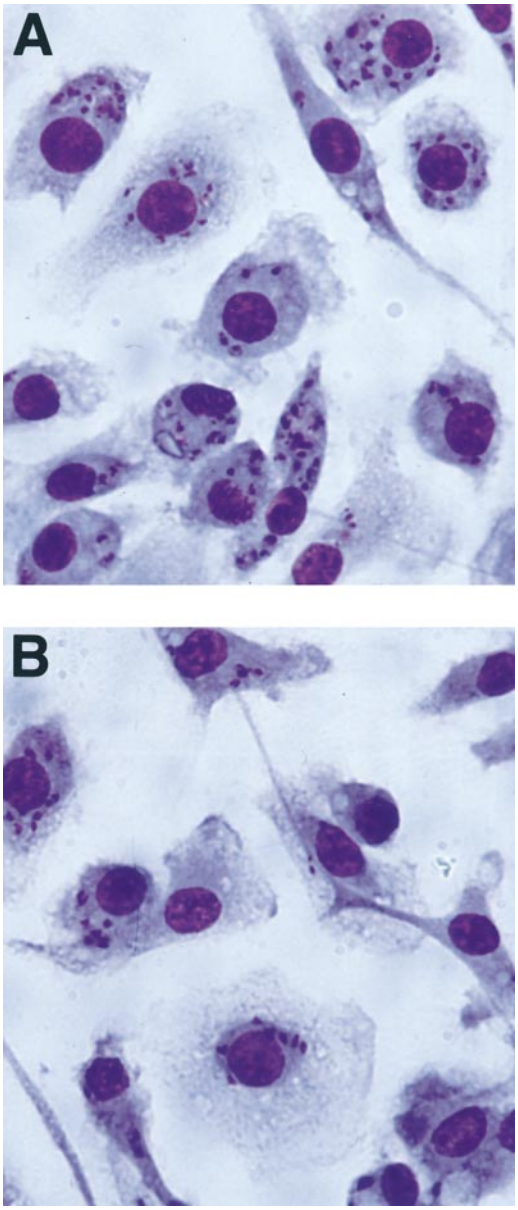

Figure 2. Micrographs from *L. infantum*–infected BALB/c macrophages in the absence (A) or presence (B) of 100 μM LOHA for 48 h. Original magnification: ×450.

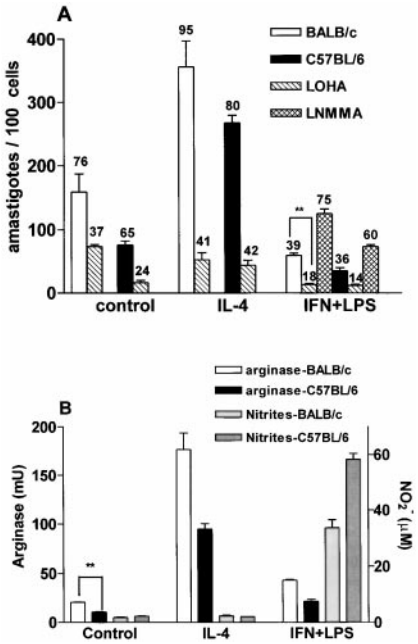

Figure 3. The susceptibility or resistance to *Leishmania* infection reflects intrinsic differences on BMMϕ-induced arginase I and NOS II. BALB/c (white bars) or C57BL/6 cells (black bars) were infected with *L. infantum* promastigotes and treated with IL-4 or IFN-γ plus LPS with or without LOHA or LNMMA. After 48 h, infected cells were used to determine intracellular parasites and cellular infection (numbers; A) or to measure arginase/nitrite values (B). $^{**}P < 0.05$ by Student's paired t test.

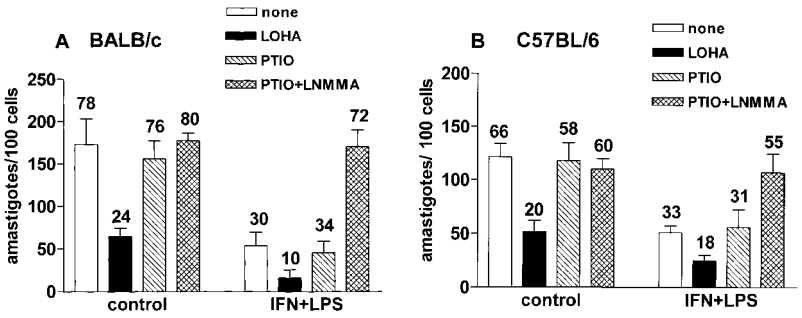

Figure 4. Scavenging of NO by carboxy-PTIO does not modify cellular infection. Macrophages from BALB/c (A) and C57BL/6 mice (B) were infected with *L. infantum* promastigotes and treated with LOHA or carboxy-PTIO with or without LNMMA, in the absence (control) or presence of IFN-γ plus LPS for 48 h. Results represent the number of intracellular parasites in 100 cells (bars) and the percentage of infected cells (numbers).

To check that LOHA was also effective in inhibiting arginase activity in our cultures, we have measured the levels of enzyme activity in infected macrophages, during the course of cellular infection. The data reveal changes in enzyme activity from 25.67 ± 1.24 in controls to 1.23 ± 0.08 in the presence of 100 μM LOHA. This suggests that the inhibition of parasite growth may be related to arginase inhibition.

LOHA induces apoptosis in tumor cell lines which are dependent on polyamine synthesis for proliferation (20, 21). Thus, the same is likely to occur in infected cultures, where the growth of parasites is prevented by inhibition of L-ornithine supply. Macrophages, a differentiated, stable population, are not dependent on polyamines for growth and therefore remain viable.

The inhibition of cellular infection can be further appreciated in the micrographs (Fig. 2) in which parasitization (Fig. 2 A) is impaired by LOHA addition (Fig. 2 B).

LOHA is a stable intermediate in the NOS reaction and can be further metabolized to other reactive nitrogen intermediates (RNIs), including NO by cellular oxidases (12). To be certain that the cytotoxic effect of LOHA was not due to NO production, we measured nitrite concentrations in culture supernatants (Fig. 1 A). LOHA did not increase nitrite levels along the dose–response curve, suggesting that NO is not involved in the antiparasitic effects of LOHA.

Macrophage Arginase I and NOS II Regulate Parasitic Growth in Opposing Ways. It is well established that there are strain-related differences with respect to resistance versus susceptibility to *Leishmania* infection: BALB/c mice develop a Th2-dominant response and become susceptible, whereas C57BL/6 mice control infection due to a predominant Th1-type response (1). Here we have compared the ability of macrophages from the two strains to control infection, depending on the fate of L-arginine metabolism. Therefore, infected cells were treated with IL-4 for arginase I induction or with IFN-γ plus LPS to have an optimal NOS II induction. As shown in Fig. 3, BMMϕ from BALB/c mice reach a higher level of infection and intracellular parasites than those from C57BL/6. In the presence of IL-4, when only arginase I is induced, both strains respond with a huge increment in parasite proliferation. However, the difference in arginase-specific activity between them is parallel to a difference in the number of total amastigotes that replicate. The opposite is the case when L-arginine is metabolized via NOS II; C57BL/6 BMMϕ have higher levels of nitrite than BALB/c cells (Fig. 3 B). Accordingly, the killing was more prominent in resistant cells compared with susceptible cells. This effect was reverted by the NOS inhibitor LNMMA (Fig. 3 A), as reported previously (16). However, in the presence of LOHA, the intracellular parasitation was almost prevented in all treated cells, including those activated with IFN-γ plus LPS, in which it was difficult to find intracellular amastigotes.

Similar results have been recently reported in macrophages from prototypical Th1 and Th2 mouse strains, which differed in their ability to divert L-arginine metabolism; macrophages from BALB/c mice mainly produced L-ornithine whereas those from C57BL/6 generated NO when induced with LPS (22). These data have supported our previous observation that L-arginine metabolism towards arginase I or NOS II determines in the macrophage different functional stages (7). Here we present evidence confirming that the final fate of L-arginine regulate the growth of *Leishmania* inside macrophages.

The NO Scavenger Carboxy-PTIO Did Not Modify Infection in the Presence of NOS II Induction. In previous experiments we added LOHA to cultured infected cells. This modified amino acid is both a substrate for NOS II (Km = 6 μM) and a product of the enzyme (23). Thus, we propose that endogenous LOHA production via NOS II could, at

Table I. *Nitrite Concentrations in Culture Supernatants of Activated-Infected BMMϕ in the Presence of LOHA and Carboxy-PTIO*

	Nitrites (μM)				
Treatment	None	LOHA	PTIO	NMMA	NMMA + PTIO
Control	1.79 ± 0.21	1.74 ± 0.23	1.81 ± 0.19	1.73 ± 0.25	1.78 ± 0.20
IFN-γ + LPS	33.56 ± 2.92	33.58 ± 2.22	65.13 ± 3.01	3.75 ± 0.29	12.03 ± 1.78

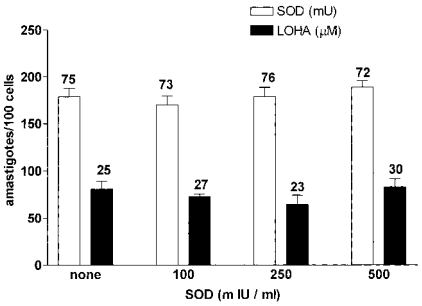

Figure 5. Effect of SOD on the inhibition of parasite infection by LOHA. BALB/c macrophages were infected with *L. infantum* promastigotes in the presence of increasing amounts of SOD alone (white bars) or together with 100 μM LOHA (black bars). After 48 h, the number of intracellular amastigotes in 100 cells (bars) and the percentage of infected cells (numbers) were counted.

least in part, be responsible for the killing of *Leishmania* in IFN-γ plus LPS–activated cells. To look at the effects of LOHA in the absence of NO, we added the NO scavenger carboxy-PTIO. This molecule oxidizes NO· to NO_2^- in a molar to molar ratio and is being used to increase the sensibility of the Griess reaction, as it generates higher levels of nitrites in culture supernatants (24). The results presented in Fig. 4 were surprising, as 100 μM of the scavenger did not revert the killing obtained by NOS II induction. The lack of effect was the same in cells from the two strains of mice, but was reverted in the presence of LMMNA plus the scavenger. However, in the same experiment, exogenous LOHA further decreased the number of intracellular parasites when added together with IFN-γ plus LPS.

Supernatant from all infected cultures were collected for nitrite determination and results presented in Table I. Although nitrite levels are not modified by exogenous LOHA, even in the presence of NOS II induction, carboxy-PTIO almost duplicates nitrite concentrations as expected. This effect was proven to be proportional to the scavenger concentration (data not shown), as described previously (24). These results suggest that LOHA is not being metabolized by NOS II, at least to enhance nitrite secretion.

It has been reported that NOS II induction in macrophages generates 20% of the total reaction products as LOHA (25). This could account for ~10 to 30 μM of this amino acid secreted into the culture media. However, it is important to note that we have not measured the real amounts of LOHA produced by activated cells. Thus, we do not know what concentration affects intracellular parasites.

In conclusion, our data suggest that the induction of NOS II in activated cells may have a double killing effect: on the one hand, the putative production of endogenous LOHA and the consequent inhibition of arginase from parasites; on the other hand, the production of RNIs that have proved to be cytotoxic against *Leishmania*. The fact that parasite killing could not be reverted by sequestering of NO does not invalidate the role of other RNIs not scavenged by carboxy-PTIO (24). Moreover, we think that NO has an undoubted role in modulating the early response to infection, inducing T cells to switch towards protective responses (3).

Effect of SOD on the Inhibition of Parasite Infection by LOHA. LOHA, when added to murine macrophages, can be the target of superoxide and yield NO as one of the reaction products (11). However, the respiratory burst, and thus, the O_2^- generation by *Leishmania*-infected cells, appears to be inefficient. The parasite has its own SOD to evade host attack (1). Moreover, previous reports have demonstrated that the killing of the parasite by NO is independent of superoxide or peroxynitrite formation (16). Nevertheless, to rule out the possible involvement of these radicals on the observed antiparasitic activity of LOHA, we added SOD. As can be seen in Fig. 5, SOD does not modify parasite killing in the presence of the inhibitor. Thus, these data suggest that the effector mechanisms of LOHA occur independently of superoxide generation.

It has been recently demonstrated that the in vivo response to *Leishmania* infection depends on a functional NOS II, but could be controlled in the absence of respiratory burst (26). Our data are in agreement with this, in the sense that NOS II induction is essential to restrain *Leishmania* infection, but point to LOHA as a new molecule, along with the RNI, directly involved in the effects of NOS II induction.

L-Ornithine Promotes Parasite Growth and Restores Cellular Infection in the Presence of LOHA. Results from Fig. 6 were made to further assess cellular infection in the absence of functional NOS II and investigate the role of L-ornithine in parasite growth. We have compared macrophages from the resistant strain C57BL/6 NOS II–deficient mice with their correspondent wild-type littermates (Fig. 6 A), and those with the ones from susceptible BALB/c mice (Fig. 6 B). In activated BMMϕ, only cells with functional NOS II are able to greatly reduce the number of amastigotes. In the

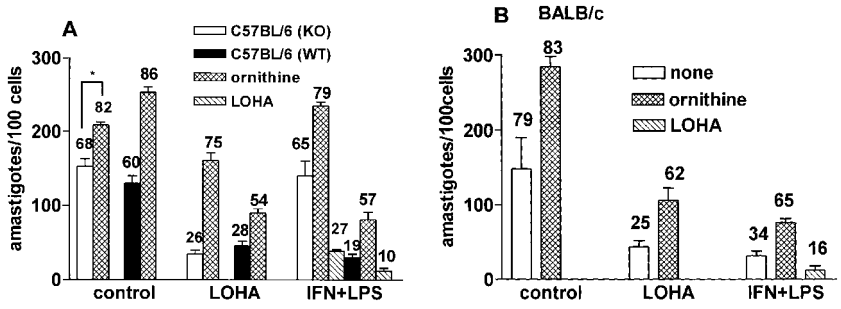

Figure 6. L-ornithine reverses the inhibitory effect of exogenous and endogenous LOHA. *L. infantum*–infected BMMϕ from NOS II knockout and C57BlL6 mice (A) or BALB/c mice (B), were untreated or treated with LOHA or IFN-γ plus LPS in the absence or presence 250 μM L-ornithine for 48 h. The results represent the number of amastigotes in 100 cells and the percentage of infection (numbers). *$P < 0.05$ by Student's paired *t* test.

presence of LOHA, the number of intracellular amastigotes was inhibited by ~75% in all infected cells. Moreover, exogenously added L-ornithine was able to significantly increase the number of intracellular parasites in macrophages from the three strains, and almost restore infection levels in the presence of LOHA (75%) and IFN-γ plus LPS (68%) treated cells. These findings strongly suggest that the inhibition of arginase from parasites is responsible for the effector mechanisms of exogenous LOHA. Furthermore, in fully activated cells, its production via NOS II could also have a role in the killing of *Leishmania* due to the reversion observed in the presence of L-ornithine.

Our results are in agreement with the recently reported affinity levels of L-arginine and LOHA to NOS II and arginase I (23). NOS II has higher affinity for L-arginine than for LOHA. In contrast, arginase I has much higher affinity for its inhibitor, LOHA, than for its substrate. Thus, despite the L-arginine concentrations present in cultures (1 mM for RPMI), LOHA can inhibit arginase from parasites and also the macrophage-induced isoform I. The result of inhibiting arginase from *Leishmania* could explain the antiparasitic effect observed in control infected cells. Whereas, by inhibiting macrophage-induced arginase I, LOHA could prevent the growth achieved in the presence of IL-4.

Therefore, the results point to a dependence of the parasitic growth on an efficient macrophage-arginase I induction; this isoform, induced in the context of a Th2-type of immune response, could optimally support the growth of *Leishmania*.

As reported before, LOHA can also be metabolized by oxidases other than NOS (12), or react with other radicals, such as H_2O_2 (27), yielding alternative products. For this reason, we do not discard the possibility that part of the effector mechanisms of LOHA could also be produced by radical generation, including NO gas not detectable by the Griess reaction. The toxic potential of NO, when exogenously applied in the form of NO donors, was efficient in killing *Leishmania* parasites in vitro (16, 28) and useful for the treatment of cutaneous leishmaniasis (29).

Polyamine synthesis is essential for normal growth and differentiation in *Leishmania* species (30). One of the main research interests in molecular parasitology of this protozoa is focused on ornithine decarboxylase gene expression and activity. Moreover, α-difluoromethylornithine (DFMO), an inhibitor of the enzyme, is being used against parasite spreading, and pentamidine, an inhibitor of arginine and polyamine transport (31), is actually the main substance for the treatment of antimony-resistant human leishmaniasis (32). This suggests that parasites with a functional arginase are sensitive to ornithine depletion. Therefore, arginase-specific inhibitors could be potential new substances of pharmacological interest for the treatment of leishmaniasis.

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