MACROPHAGE KILLING OF *LEISHMANIA AMAZONENSIS* AMASTIGOTES REQUIRES BOTH NITRIC OXIDE AND SUPEROXIDE

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Abstract. The requirements for effective and efficient intracellular killing of *Leishmania amazonensis* by activated macrophages are unknown. Despite resistance to the arginase inhibitor LOHA by intracellular *L. amazonensis* amastigotes, enhanced replication did not account for the relative resistance of this parasite to macrophage activation. Herein we report that the presence of both superoxide and nitric oxide is necessary for efficient killing of *L. amazonensis* amastigotes within LPS/IFN-γ-activated bone marrow-derived macrophages generated from C3H mice. Addition of an extracellular signal-regulated kinase (ERK) inhibitor to *L. amazonensis*-infected macrophages increased the ability of these activated macrophages to kill *L. amazonensis* amastigotes. This enhanced macrophage killing through addition of ERK inhibitor was abrogated by inhibition of superoxide or iNOS, whereas inhibiting superoxide had no effect on the killing of *L. major*. These results suggest that ERK activation may modulate effective macrophage killing, leading to the ability of *L. amazonensis* to resist elimination within activated macrophages.

INTRODUCTION

Upregulation of inducible nitric oxide synthase (iNOS) and subsequent nitric oxide production (NO) is important for parasitic activity of infected macrophages during experimental infection of mice with *Leishmania* spp.1-3 Evidence indicates that the anti-parasitic activity of this enzyme involves generation of reactive nitrogen intermediates, such as nitric oxide and/or other reactive nitrogen molecules.4 These reactive nitrogen species may, in turn, interact with NADPH-oxidase dependent superoxide to produce or enhance cytotoxic responses.5 Recent reports suggest that the contribution of NADPH-oxidase-dependent superoxide production to a leishmanicidal response may vary between different cell types and stage of infection.2,6 In addition to nitric oxide production, the iNOS generated arginine metabolite Nω-hydroxy-L-arginine (LOHA) has been shown to limit growth of *Leishmania* within infected macrophages and play a significant role in limiting the in vitro infection rate.7

Parasite persistence within macrophages is determined by a balance between the ability of the immune response to sufficiently activate *Leishmania*-infected macrophages versus the ability of the parasite to resist cytotoxic mechanisms of macrophage activation. The outcome of leishmaniasis in vivo has been shown to depend on the induction of a CD4+ Th1 response, although the disparate survival phenotype of *L. amazonensis* and *L. major* parasites within genetically-identical hosts may involve more than differences in the host T-cell response alone. We have recently shown that, in contrast to *L. major*, lymphocyte-induced killing of *L. amazonensis* amastigotes in vitro required antibody and superoxide production.8 The ability to inhibit and/or resist macrophage activation has been described for a variety of *Leishmania* species.9 Host intracellular signaling pathways are specifically targeted by *Leishmania* parasites during infection to prevent a productive immune response. *L. major* promastigote lipo-phosphoglycan has been shown to inhibit macrophage IL-12p40 production through extracellular signal-regulated kinase (ERK) activation.10 *L. major*-infected macrophages produce limited CD40-induced expression of IL-12p40 as a result of enhanced IL-10 production mediated through the host ERK pathway.11 Although a variety of *Leishmania* spp. including *L. amazonensis*9,12 have been shown to inhibit and/or resist macrophage activation, the requirements for effective intracellular *L. amazonensis* killing by activated macrophages and the mechanisms involved remain unknown.

We have focused our studies of *L. amazonensis* infection on the C3H mouse strain as these mice are susceptible to infection by this parasite and yet resistant to the related parasite *L. major*. In particular, we have found that a previous infection with *L. major* will limit disease upon subsequent infection with *L. amazonensis*, indicating that C3H mice can upregulate an effective anti-*L. amazonensis* immune response.13 In this current work, we examine the ability of *L. amazonensis* amastigote parasites to resist defined promacrophage activation conditions (LPS and IFN-γ) after infection of non-inflammatory bone marrow-derived macrophages (BMM). We found that *L. amazonensis* amastigote parasites were not killed as efficiently as *L. major* amastigotes despite equivalent iNOS and nitric oxide levels.

To further understand this phenomenon of *L. amazonensis* amastigote resistance to macrophage activation, we assessed the role of parasite replication in this resistance using LOHA and hydroxyurea. We also determined that MAPK ERK activation promoted an inhibition of macrophage function because ERK inhibition promoted intracellular killing of *L. amazonensis*. We used this phenomenon to determine the relative contribution of superoxide and nitric oxide to the parasiticial response. The results of this analysis highlight the differences required for intracellular killing of disparate *Leishmania* species, confirming that the definition of an adequate host immune response against one *Leishmania* species does not apply to all parasites of this genus.

MATERIALS AND METHODS

Mice. C3HeB/FeJ and C3Smn.CB17-Prkda<sup>scid</sup>/J (C3H scid) mice were obtained from Jackson Laboratory (Bar Harbor, Maine). Mice were maintained in a specific-pathogen-free facility. For the propagation of lesion-derived amastigotes, C3H SCID mice were infected with 5 × 10<sup>6</sup> stationary-phase pro-
mastigotes in 50 μL of PBS in the left hind footpad. All procedures involving animals were approved by the Committee on Animal Care at Iowa State University and complied with the National Institutes of Health guidelines for the humane use of laboratory animals.

**Parasites.** *L. major* (MHOM/IL/80/Freidlin) and *L. amazonensis* (MHOM/BR/00/LTB0016) promastigotes were grown to stationary phase in Grace’s insect culture medium (Life Technologies, Gaithersburg, MD) with 20% heat-inactivated FCS, 2 mM glutamine, 100 U penicillin per mL, and 100 μg of streptomycin per mL. Amastigotes that were used for in vitro infection were tissue-derived and were harvested from lesions of C3H SCID mice as described.\(^1\)

**Cells and cell culture.** Cells were obtained from the bone marrow of the mouse femur and tibia (15–20 × 10⁶ cells) and plated in a 150 × 15 mm Petri dish with 30 mL of macrophage medium containing 30% L-cell conditioned medium, 20% FCS, and 50% Dulbecco’s modification of Eagle’s medium (DMEM), 2 mM glutamine, 100 U penicillin per mL, 100 μg of streptomycin per mL, and 1 mM sodium pyruvate at 37°C and 5% CO₂. After 2 days, another 20 mL of macrophage medium was added to the Petri dish. At Day 7, the non-adherent cells were removed and the plate was scraped to harvest the adherent cell population. After washing with PBS, live cells were counted using trypan blue exclusion and resuspended in complete tissue culture medium (CTCM) containing DMEM, 10% FCS, 2 mM glutamine, 100 U penicillin per mL, 100 μg of streptomycin per mL, 25 mM 4-(2-hydroxyethyl)piperazine-1-ethane sulfonic acid (HEPES), and 0.05 μM 2-mercaptoethanol.

**Macrophage infection and activation.** BMM were plated in 24-well plates with glass cover slips at a rate of 5 × 10⁴ cells/well, in 1 mL of CTCM. After 24 hours, the BMM were infected with either *L. major* or *L. amazonensis* amastigotes at a 3:1 ratio and incubated at 34°C for 24 hours. The wells were washed twice with DMEM to remove extracellular amastigotes, and a final volume of 1 mL of CTCM was added. The macrophages were then left non-activated or activated with 100 U/mL INF-γ and 100 ng/mL LPS (Escherichia coli J5 from Sigma-Aldrich, St. Louis, MO). For replication-inhibition experiments, a concentration of 500 μM N⁶-hydroxy-4-aminobutyric acid (N⁶-HA)-arginine (LOHA, Calbiochem, La Jolla, CA) or 4 mM hydroxyurea (Alfa Aesar, Ward Hill, MA) were used in each well of both activated and non-activated infected macrophages. Also, 15 μM Mn(III)tetakis(4-benzoic acid)porphyrin chloride (MtTBP, BIOMOL, Plymouth Meeting, PA), 1 mM L-N⁶-(1-iminoethyl)lysine (L-NIL, A.G. Scientific, San Diego, CA) or 20 μM 2‘,-amino-3‘-methoxyflavone (PD98059, Alexis, Lausen, Switzerland) were added at the time of activation. The cover slips were removed as indicated after 1, 2, or 3 days post-activation, stained using the nonspecific HEMA 3 stain set (Fisher Diagnostics, Middletown, VA), and mounted on glass slides. All added compounds, except PD98059, were readily soluble in water or medium. PD98059 was dissolved in dimethyl sulfoxide (DMSO). There was no statistical difference in the rate of infection of non-activated and activated cultures compared with DMSO solvent controls (data not shown).

**Determination of infection rate of macrophages and parasite count.** Each individual cover slip was counted via light microscopy by examining three areas using the 100× objective. In each area, 100 macrophages were examined, and the number of infected macrophages and number of parasites in each macrophage were counted. An average of three areas was used to determine the percent of infected macrophages and number of parasites per 100 macrophages for each cover slip.

**Determination of nitric oxide.** Nitrite concentrations were determined using Greiss reagent as described previously.\(^1\) Briefly, equal volumes (50 μL) of cell culture medium and Greiss reagent (LabChem, Pittsburgh, PA) were mixed and incubated at room temperature, and absorbance was measured at 550 nm with a microplate reader (Molecular Devices, Sunnyvale, CA). Nitrite concentration was determined using a standard curve generated with sodium nitrite.

**Western blot.** Twenty-four hours after activation, macrophages were washed two times with 1 mL of PBS, 100 μL of washing buffer was added (0.32 M sucrose, 3 mM calcium chloride, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM tris(hydroxymethyl)aminomethane (Tris, pH 8.0), 5 mM sodium fluoride, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 0.1 mM sodium orthovanadate, and 20 μL per mL protease inhibitor cocktail (Sigma, St. Louis, MO)). Lysis buffer (100 μL) was added next (wash buffer plus 1% Nonidet P-40). The suspension was centrifuged at 2500g, and the supernatant was stored at –80°C. Protein concentrations were measured via BCA (bicinchoninic acid) protein assay (Pierce, Rockford, IL) using a BSA standard. Cytoplasmic extracts were separated using 8% PAGE and transferred to nitrocellulose membrane using a semi-dry blotting apparatus from Bio-Rad (Hercules, CA). The membrane was blocked with 5% nonfat dry milk in PBS 0.1% Tween. Rabbit polyclonal anti-mouse iNOS (Upstate, Lake Placid, NY) was hybridized to the membrane overnight at 4°C at a 1:5000 dilution. The membrane was washed and hybridized to a secondary goat anti-rabbit antibody conjugated to hydrogen peroxidase (Jackson ImmunoResearch, West Grove, PA) at a 1:50,000 dilution. Signal was detected using the Pierce Supersignal Reagents (Pierce, Rockford, IL) as directed by the manufacturer. Membranes were rehybridized with a rabbit polyclonal anti-mouse actin antibody at a 1:5000 dilution (Sigma) as described above. For ERK phosphorylation analysis, cell extracts were prepared as above and run on 12% SDS-PAGE followed by transfer to PVDF membrane. Membranes were probed with either antibody and chemiluminescene (Pierce Supersignal). Relative phospho-ERK activation levels were assessed by normalizing to the total levels of actin under the same condition (data not shown).

**ELISA for IL-10.** The level of IL-10 protein in supernates was determined by ELISA using antibodies from clone JES5-2A5 as the capture antibody and biotinylated antibodies from clone SXC-1 as detection antibody according to the manufacturer’s instructions (Pharmingen, San Diego, CA).

**Statistics.** Data analysis was performed by using StatView 5.0.1 (SAS, Inc., Cary, NC). Statistical significance was determined by the Scheffe test for pairwise comparisons when comparing between different days or various treatments and activation conditions. A paired t test was used when comparing identical parasite species and cultures under identical activation conditions that varied only by a single treatment, as indicated in the figure legend.
RESULTS

Parasite replication plays a minimal role in differential \textit{L. amazonensis} amastigote survival in vitro. To determine whether \textit{L. amazonensis} amastigotes were resistant to killing by macrophages, BMM derived from C3H mice were infected with \textit{L. amazonensis} or \textit{L. major} amastigotes and stimulated 24 hr later with LPS and IFN-\textgamma. A significant reduction in number of infected macrophages after activation was detected after 3 days of activation for \textit{L. major} parasites (Figure 1B, LM C). Furthermore, a comparison between the percentage of infected macrophages under non-activated and activated conditions at Day 3 demonstrated a disparate parasite survival rate at 3 days post-stimulation; differences in percent \textit{L. amazonensis}-infected macrophages after activation was not significant (Figure 1C, C), whereas differences in percent \textit{L. major}-infected macrophages was significant (Figure 1D, C).

Several studies have demonstrated that parasite replication significantly influences infection rate of in vitro cultures. LOHA, an intermediate of NO generation by iNOS and an inhibitor of arginase activity, has been shown to inhibit \textit{L. major} and \textit{L. infantum} parasite replication.\textsuperscript{7} Therefore, we wanted to determine the ability of LOHA to influence the ability of \textit{L. amazonensis} to persist within macrophages. As expected, after LOHA treatment, \textit{L. major} was found to be sensitive to LOHA as there was a significant decrease in the percent of macrophages infected with \textit{L. major} under non-activating conditions (Figure 1D, black bars, C, and LOHA). After LOHA treatment, there was no significant decrease in the infection rate of \textit{L. amazonensis}-infected macrophages, either with or without activation (Figure 1C, C and LOHA).

To more closely evaluate the effect of LOHA on \textit{L. amazonensis} replication, we used hydroxyurea, a known inhibitor of DNA synthesis, as a positive control for inhibiting parasite replication. This compound has been shown to halt intracellular replication of \textit{L. amazonensis}.\textsuperscript{16} In non-activated macrophages, both \textit{L. amazonensis} and \textit{L. major} amastigotes were found to replicate during the observed 3 days post-activation as measured by the number of parasites per 100 macrophages (Figure 2A, LA C and LM C). Replication of \textit{L. major} amastigotes was not detected in the presence of LOHA (Figure 2A, LM LOHA). In contrast, the replication of \textit{L. amazonensis} amastigotes was not limited by LOHA administration (Figure 2A, LA LOHA). Hydroxyurea treatment significantly limited both the numbers of \textit{L. major}-infected macrophages (Figure 1D, C and HDU) and replication of \textit{L. major} (Figure 2A, LM HDU) under non-activated conditions similar to the effect observed after LOHA treatment and reconfirmed a role for parasite replication in influencing the rate of \textit{L. major}-macrophage infection in our system. Hydroxyurea reduced \textit{L. amazonensis} replication, as there was no significant increase in the number of parasites per 100 macrophages infected with \textit{L. amazonensis} under non-activating conditions (Figure 1D, black bars, C, and LOHA).

![Figure 1](image_url)

**Figure 1.** \textit{L. amazonensis} amastigotes are resistant to killing by LPS/IFN-\textgamma activated macrophages. C3HeB/FeJ BMM were infected with \textit{L. amazonensis} (black) or \textit{L. major} (gray) amastigotes and left unactivated (A) or activated 24 hr later with IFN-\textgamma and LPS (B) and either mock treated (circles) or treated with 500 \textmu M LOHA (squares) or 4 mM hydroxyurea (HDU, triangles). On Days 1, 2, and 3 post-treatment, the percent macrophages infected with \textit{L. amazonensis} or \textit{L. major} was determined as described in Materials and Methods. The percent-infected macrophages at Day 3 are plotted under all treatments for \textit{L. amazonensis} (C) and \textit{L. major} (D) infections. C = mock treated, HDU = hydroxyurea, *P < 0.05 between days (A, B) and between control and treated groups (D), #P < 0.05 between activation conditions (C, D), Scheffe test. Data shown are the mean ± SE from 3 independent experiments.
ropages over the 3 days of measurement (Figure 2A, LA HDU). However, even in the absence of replication, there was no significant change in the percentage of infected macrophages under non-activated conditions (Figure 1C, C and HDU). There was a significant reduction in the percentage of hydroxyurea-treated activated macrophages that were infected with *L. amazonensis* amastigotes when compared with treated non-activated controls (Figure 1C, HDU). This reduction was small relative to that of *L. major* infected macrophages either with or without replication inhibitors (Figure 1D). In summary, LOHA does not limit *L. amazonensis* replication in culture, but as limiting replication with hydroxyurea treatment does not lead to a large relative reduction in the number of infected macrophages, *L. amazonensis* replication is unlikely to be the predominant force behind the inability of macrophages to kill intracellular *L. amazonensis*.

**iNOS does play a role in determining the survival rate of *L. amazonensis* amastigotes.** The preceding results indicate that *L. amazonensis* resistance to macrophage killing is not dependent on species-specific differences in resistance to LOHA-mediated inhibition of parasite replication between *L. amazonensis* and *L. major* in this assay but instead suggests that *L. amazonensis* amastigotes may be resistant to nitric oxide-mediated death. To begin to evaluate this hypothesis we measured levels of nitrite production on the 3 days post-activation as well as iNOS protein expression. We found no significant differences in the levels of nitrite accumulation on each day after activation (Days 1 and 2, data not shown; Day 3, *L. amazonensis* 51.91 ± 13.37 μM, *L. major* 62.40 ± 12.67 μM, mean ± SEM). Furthermore, Western blot analysis confirmed equivalent iNOS protein expression between uninfected, *L. amazonensis*-infected, and *L. major*-infected macrophages at 24 hours post-LPS/IFN-γ activation (data not shown). These results support the conclusion that differences in the ability to produce nitric oxide does not determine differences in the ability of activated-macrophages to kill *L. major* and *L. amazonensis*. These results are consistent with previous experiments using *L. amazonensis* promastigotes.18 The regulatory cytokine IL-10 has been shown to inhibit macrophage activation and is produced in response to macrophage infection with other *Leishmania* parasites under a variety of experimental conditions.19–20 We found low levels of IL-10 that did not significantly differ between cultures of *L. amazonensis*-infected (1.14 ± 1.04 ng/mL, mean ± SEM) and *L. major*-infected macrophages (2.60 ± 2.53 ng/mL, mean ± SEM). To determine if nitric oxide was playing any role in limiting the *L. amazonensis* infection in vitro, we inhibited iNOS activity with a specific inhibitor, l-NIL (1 mM). As the initial findings here conclude that parasite replication is not a major contributor to differences seen in percent infected macrophages, we focused on Day 3 post-activation, the time when *L. major* demonstrates significant activation-induced reductions in the infection rate compared with the non-activated controls (Figure 1D). As expected, l-NIL abrogated *L. major* killing (Figure 3, black). However, the relatively small reduction in the percentage of *L. amazonensis* infected macrophages observed after activation was also completely abrogated (Figure 3, gray), and the infection rate reached a level higher than the infection rate of unactivated cells. These findings indicate that, after activation, iNOS activity does play some role in limiting the number of macrophages infected with *L. ama-

![Figure 2](image2.png)

**Figure 2.** *L. amazonensis* amastigotes are resistant to the arginase inhibitor N′-hydroxy-nor-l-arginine (LOHA) and sensitive to hydroxyurea. C3HeB/FeJ BMM were infected and at 24 hr post-infection left non-activated (A) or activated (B) as described in Figure 1 and assayed for the number of parasites per 100 macrophages as described in Materials and Methods. Either 500 μM LOHA (squares) or 4 mM hydroxyurea (triangles) was added to the appropriate wells 24 hr post-infection. The data shown are Days 1 to 3 post-treatment and activation (*P* < 0.05 for differences between days, Scheffe). Results shown are the mean ± SE of 3 independent experiments.

![Figure 3](image3.png)

**Figure 3.** *L. amazonensis* amastigotes are partially resistant to NO mediated killing. C3HeB/FeJ BMM were infected with *L. amazonensis* or *L. major* amastigotes and left unactivated or activated 24 hr later with IFN-γ and LPS as described in Figure 1 and mock treated (activated) or treated with 1 mM l-NIL (activated + l-NIL). On Day 3 post-treatment, the percent macrophages infected with *L. amazonensis* or *L. major* was determined as in Materials and Methods. % of control = (percent infected macrophages with activation/percent infected macrophages in the corresponding non-activated culture) × 100. Data shown are the mean ± SE from 3 independent experiments. *Significant difference (*P* < 0.05, paired t test).
zonensis amastigotes, although this pathway alone is not sufficient to promote a reduction in the rate of infection of L. amazonensis to levels comparable to L. major.

**ERK inhibitor PD98059 promotes superoxide-dependent killing of L. amazonensis in activated macrophages.** Recently, *Leishmania* has been shown to limit macrophage function through activation of the mitogen-activated protein kinase (MAP kinase) ERK either with or without concomitant production of macrophage-derived IL-10.\(^{10,11}\) Therefore, we wanted to test whether the ERK signaling pathway could play a role in limiting macrophage responses after *L. amazonensis* amastigote infection in vitro. Killing of *L. amazonensis* significantly increased in the presence of the ERK inhibitor PD98059 (20 μM) (Figure 4A). This reduction in infection was similar to that seen in *L. major*-infected activated macrophages. ERK inhibitor alone did not affect the infection rate of non-activated macrophages for either parasite (data not shown). ERK activation was associated with LPS/IFN-γ treatment of *L. amazonensis*-infected BMM as demonstrated by detectable ERK phosphorylation at 30 minutes post-activation. Treatment of these cells at the time of activation with the inhibitor PD98059 reduced the level of ERK phosphorylation (Figure 4B). Because we demonstrated no differential increase in iNOS or NO microbicidal pathways between *L. amazonensis* and *L. major* in our in vitro system, we hypothesized that other effector molecules would be enhanced in the presence of PD98059. Therefore we co-treated the infected activated cells with a cell-permeable superoxide dismutase mimetic MnTBAP (15 μM) to scavenge superoxide and l-NIL (1 mM) to inhibit iNOS. As seen in Figure 4A, enhanced macrophage killing of *L. amazonensis* established by the ERK inhibitor was negated in the presence of the superoxide inhibitor but had minimal effect on *L. major* killing. As expected from Figure 3, both *L. amazonensis* and *L. major* killing was inhibited after iNOS and ERK inhibition (Figure 4A). Neither MnTBAP nor l-NIL alone has any effect on the macrophage infection rate in the absence of activation (data not shown). These results demonstrate that addition of ERK inhibitor PD98059 may promote superoxide- and iNOS-dependent killing of *L. amazonensis* parasites in infected activated macrophages.

**DISCUSSION**

Intracellular BMM reduction of amastigote stage *L. amazonensis* parasites after activation requires both nitric oxide and superoxide production. Other studies have demonstrated NO-dependent killing of *L. amazonensis* in activated macrophages\(^{17}\) as determined by enhanced parasite DNA fragmentation.\(^{21}\) However, the relative resistance of *L. amazonensis* amastigotes to IFN-γ/LPS-mediated macrophage activation was not dependent on suppression of nitrite production or iNOS (data not shown). The cellular and molecular targets of iNOS-mediated leishmanicidal activity are somewhat controversial; NO is not thought to act alone during a productive leishmanicidal response.\(^{3}\) Inhibition of iNOS by l-NIL increased the infection rate of activated macrophages for both parasites (Figure 3), agreeing with previous work that demonstrated a role for iNOS in controlling *Leishmania* in vivo\(^{1–3}\) and in vitro.\(^5,22\) Our results support the evidence that NO acts as a cytostatic agent against *L. amazonensis*, indicating that other factors, including superoxide (Figure 4), are needed to kill the amastigote stage of the parasite. The enhanced macrophage infection rate above control levels after l-NIL treatment (120%, Figure 3) is consistent with recent data indicating that IFN-γ can act as a growth factor for *L. amazonensis* within macrophages. In the absence of cytostatic NO, macrophage activation with IFN-γ might actually promote parasite growth and significantly enhance the rate of infection in vitro.\(^5\) Superoxide is a necessary component of peroxynitrite, shown to be more potent in killing *L. amazonensis* amastigote parasites in vitro.\(^4\) In macrophages, peroxynitrite and its derivatives are thought to be generated primarily from the close association of NO and superoxide.\(^5\)
Contradictory reports have been shown for the role of MAP kinases during *Leishmania* spp. infection of macrophages. Several studies have indicated that limited ERK phosphorylation correlates with infection as both *L. amazonensis* amastigotes and *Leishmania donovani* promastigotes have been shown to prevent ERK activation and *L. mexicana* has been shown to promote ERK degradation.\(^{24-27}\) On the other hand, ERK signaling has been associated with both IL-10-dependent and -independent limiting of both IL-12p40 and iNOS production.\(^{10,11}\) Here we show that the ERK inhibitor, PD98059, reduced the survivability of intracellular *L. amazonensis* in BMM via superoxide- and NO-dependent mechanisms (Figure 4A). Absence of differential expression of IL-10 in our system between *L. major* and *L. amazonensis* infection, as determined by ELISA, was expected based on previously observed findings that IL-10 has been shown to inhibit production of both iNOS and NO.\(^{19,28}\) As neither iNOS nor NO is differentially produced between *L. amazonensis* and *L. major* infection, regulatory IL-10 production was also unlikely to be significantly different between these infections. Although we have not yet explored the actual relationship between superoxide production and ERK signaling in our experimental system, it has been previously shown that LPS-activated peritoneal macrophages induce Cu/Zn superoxide dismutase production through ERK activation.\(^{29}\) These results support previous studies indicating a need for peroxynitrite to control *L. amazonensis* as a result of both superoxide and NO production.\(^{30}\) Although this relationship may also occur in macrophages infected with *L. major* amastigotes, we have demonstrated that the killing of *L. major* within activated macrophages is more directly related to the presence of nitric oxide rather than the presence of superoxide (Figure 4A).

Recent studies have demonstrated not only that iNOS-generated NO is important for leishmanicidal activity but also that LOHA (an intermediate of iNOS-dependent NO generation) can have a direct effect in inhibiting the growth of *Leishmania*.\(^{7}\) These previous studies demonstrated that both *L. major* and *L. infantum* were susceptible to the inhibitory effects of this compound at a concentration of 100 \(\mu M.\)\(^{7}\) We found that, in contrast to *L. major*, there was no growth inhibition of *L. amazonensis* up to a LOHA concentration of 500 \(\mu M\) (Figure 2). Parasite replication has been previously demonstrated to correlate positively with the level of *L. major* infection using in vitro macrophage infection rates.\(^{7}\) Surprisingly, inhibition of parasite replication with hydroxyurea had only modest effects on limiting *L. amazonensis* infection and survival in activated macrophages (Figure 2). Lack of further reduction in parasites per 100 macrophages after hydroxyurea treatment in activated cells would suggest that there is no significant replication occurring in these cells. This further supports previous studies that indicated that NO is cytostatic for *L. amazonensis* but not cytotoxic.\(^{4}\) Enhanced replication of *L. amazonensis* was not responsible for disparate parasite counts (Figure 2).

The studies presented herein indicate that differential *L. amazonensis* amastigote resistance to host-mediated anti-leishmanial responses in non-inflammatory macrophages from C3H mice activated with LPS and IFN-\(\gamma\) may be mediated through ERK inhibition of superoxide killing. We have found that, although *L. amazonensis* is resistant to the growth inhibitory effects of LOHA, the parasite does not require enhanced replication for increased survival within activated macrophages. To defeat these mechanisms of host resistance, a successful anti-leishmanicidal response to *L. amazonensis* amastigotes requires both superoxide and NO production. This result is consistent with our previous studies indicating that superoxide is required for killing intracellular *L. amazonensis* in an in vitro assay using lymphocytes from *L. major*-infected animals.\(^{8}\) These studies implicate ERK as a host-signaling molecule potentially central to influencing the host-pathogen relationship of *L. amazonensis*, warranting further study. These findings highlight the differences required for intracellular killing of disparate *Leishmania* species and support recent studies demonstrating a unique host-parasite relationship for *L. amazonensis*.\(^{8,23}\) confirming that that the definition of an adequate host immune response against one *Leishmania* species does not apply to all parasites of this genus.

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