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Original article

# Differential properties of CBA/J mononuclear phagocytes recovered from an inflammatory site and probed with two different species of *Leishmania*

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**Abstract**

While CBA/J mice fail to be permissive to *Leishmania amazonensis*-driven pathogenic processes, they heal easily following *Leishmania major* infection. The early-phase events are crucial to the outcome of *Leishmania* infection and it is known that macrophages (Mϕ) are important in infection control. In the present study we investigated the role of Mϕ in driving CBA/J susceptibility to *L. amazonensis*. We performed kinetic studies and compared the capacity of *L. amazonensis* and *L. major* to infect Mϕ. There was no difference in percentages of infection or parasite burden for 6 h between the two groups. In contrast, after 12 h we observed that infection was about twice as high in *L. amazonensis*- than in *L. major*-infected Mϕ. In addition, rIFN-γ added to the cultures induced nitric oxide (NO) production, and did not modify *L. amazonensis* infection, although the percentage of *L. major* infection was significantly reduced. This reduction in *L. major* infection is a TNF-α dependent mechanism as *L. major*-infected Mϕ expressed twice as much TNF-α mRNA as *L. amazonensis*-infected cells, and anti-TNF-α reversed the IFN-γ effect. Moreover, rTNF-α plus IFN-γ were able to significantly reduce the percentage of *L. amazonensis*-infected cells but not to the same extent as in *L. major* infection. Despite having higher NO production than IFN-γ-treated cells, AMG addition to IFN-γ-plus TNF-α-treated cells only partially reversed the inhibition in *L. major*, but not in *L. amazonensis* infection. Thus, in this study, we demonstrated that *L. amazonensis* both inactivated and resisted innate and IFN-γ-induced Mϕ killing mechanisms, indicating that the nature of the parasite and its interaction with Mϕ could determine immune response polarization.



*Keywords:* Macrophages; CBA/J mice; *L. amazonensis*

## 1. Introduction

Leishmaniases are a group of diseases caused by the trypanosomatidae *Leishmania* sp. affecting about 2 million people every year in Africa, Asia, South America and the Mediterranean area. In humans its development manifests either as visceral or cutaneous diseases, as well as asymptomatic processes. Cutaneous leishmaniasis is caused mainly by *L. major* in the Old World [1] and *L. amazonensis* and *L.* (*Viannia*) *braziliensis* in the New World [2].

Experimental leishmaniasis has been widely used to characterize the immune response against *Leishmania* parasites. Most studies have been performed comparing strains of mice of different genetic backgrounds that are susceptible or resistant to *L. major* infection [3–5]. Resistance and susceptibility to infection depend on the development of specific CD4+ T helper (Th)-1 or Th-2 responses, respectively [6]. We have recently developed an in vivo mouse model and demonstrated that while CBA/J mice fail to acquire *L. amazonensis* infection, they recover following infection with *L. major* [7]. These results emphasize the importance of parasites in driving the host immune response. *L. amazonensis* induced lesions showing a monomorphic infiltrate of highly parasitised and large vacuolated macrophages (Mϕ) [7]. Lymph node (LN) cells from CBA/J mice infected by *L. major* produced large amounts of gamma interferon (IFN-γ) when restimulated in vitro with *Leishmania* promastigotes, whereas infec-

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tion by *L. amazonensis* led to interleukin (IL)-4 production by LN cells from infected mice [7]. The first cellular events, which could elucidate these differences, are unknown.

There is considerable evidence of the importance of the innate immune response in the outcome of *Leishmania* infection [8–13]. Mϕ play an important role against *Leishmania*, since they are the principal host cell and they present antigens to specific Th cells [14,15]. In addition, they produce cytokines which drive Th-1 and Th-2 responses, modulating the immune responses and influencing intracellular parasite survival [16]. Mϕ produce nitric oxide (NO) and/or oxygen radicals, which are toxic to intracellular parasites [17–21]. The importance of Mϕ in the fate of *Leishmania* infection has been demonstrated by studies in vitro using Mϕ from mice with different susceptibilities to infection by *Leishmania*. Thus, Mϕ from resistant mice inhibit the proliferation of *L. major* amastigotes [22,23] and are easily activated by T cell-derived cytokines [24,25]. Furthermore, Mϕ from susceptible mice have an impaired response to signal activation after *L. major* infection [26]. In this report we investigated the role of CBA/J Mϕ on the determination of the differences observed between in vivo *L. amazonensis* and *L. major* infection [7]. We tested whether CBA/J susceptibility to *L. amazonensis* infection is related to parasite resistance to Mϕ killing mechanisms, or whether *L. amazonensis* was unable to induce Mϕ activation to kill intracellular amastigotes. We established an in vitro model to compare the ability of *L. amazonensis* and *L. major* to infect and activate CBA/J Mϕ in vitro. We also presented evidence of whether Mϕ from this mouse strain were activated or not in the presence of IFN-γ and whether they were able to kill *L. major*, but not *L. amazonensis*.

## 2. Materials and methods

### 2.1. Mice

Male and female CBA/J mice, 6–12 weeks old, were provided by the Central Animal Facility of the IOC/FIOCRUZ and from the Animal Facility of CPqGM/FIOCRUZ. These animals were housed under specific-pathogen-free conditions and fed on commercial ration and water ad libitum.

### 2.2. Parasites

Promastigotes in stationary phase used in this work were *L. amazonensis* (MHOM/Br88/Ba-125) and *L. major* (MHOM/RI/-/WR-173) provided by Dr. Aldina Barral, from the Laboratory of Immunopathology of CPqGM/FIOCRUZ. Fresh *L. amazonensis* or *L. major* promastigotes were derived from isolated amastigotes from LNs of C57BL/6 resistant mice, resuspended in Novy-Nicolle-MacNeal blood agar and then transferred to total LIT plus 10% fetal bovine serum (FBS) (Cultilab, Campinas, Brazil) for a maximum of six passages. For Mϕ experiments, the promastigotes were expanded for 3–5 d until reaching the stationary phase, washed with saline, and then adjusted to the desired concentrations, indicated in the results. According to procyclic form susceptibility to be lysed by complement present in fresh serum, the percentage of metacyclic forms was determined by incubating the promastigotes at stationary phase with different concentrations of fresh serum for 30 min at 37 °C [27]. Logarithmic phase cultures were used as control, and we observed that all promastigotes were lysed in fresh serum-containing medium. The number of live parasites in stationary-phase cultures was counted by optical microscopy, and we obtained similar amounts of complement-resistant promastigotes in levels of 50% for both *L. amazonensis* and *L. major* stationary promastigotes.

### 2.3. Mϕ culture

Thioglycolate-induced peritoneal exudate cells (PEC Mϕ) were prepared from the peritoneal cavity of CBA/J mice 3–4 d after intraperitoneal injection of 2.5 ml 3% thioglycollate medium (Sigma Chemical, St. Louis, MO). PEC Mϕ were obtained by flushing the peritoneal cavity twice with 10 ml ice-cold saline plus heparin (20 UI/ml) and centrifuged at 300 × *g* for 10 min. The cells were resuspended in DMEM supplemented with 25 mM HEPES (*N*–2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) pH 7.4, 2 mM glutamine, 100 UI/ml penicillin, 100 µg/ml streptomycin, 2.0 g/l sodium bicarbonate plus 10% FBS (Gibco Laboratories) (complete medium). Mϕ ($2 \times 10^5$ or $1 \times 10^6$ per ml) in complete medium were plated in 24-well culture plates (Costar, Cambridge, MI) containing or not 13-mm diameter glass coverslips (Glasstécnica Imp. São Paulo, BR) at 37 °C in 5% $CO_2$/95% humidified air. After 4 h, non-adherent cells were washed three times with RPMI-1640 supplemented with 25 mM HEPES. Afterwards, Mϕ were incubated in fresh medium with different stimuli.

### 2.4. Mϕ infection

Mϕ were infected with *L. amazonensis* or *L. major* promastigotes in stationary phase, at ratio 10:1. After 90 min, 3, 6 or 12 h, cells were washed and fixed. In some experiments, cells were washed to remove non-internalized parasites after 12 h of infection, at time zero (0), and reincubated for an additional 24, 48 and 72 h after infection. The cells were fixed with methanol or 99.3% ethanol for 10 min and stained with hematoxilin and eosin. The percentage of infected cells and the *Leishmania* number per Mϕ were determined by light microscopy observations (magnification, ×1000), and at least 200 Mϕ were counted per coverslip.

### 2.5. Hydrogen peroxide determination

$H_2O_2$ was measured by the peroxidase-dependent oxidation of phenol red [28]. The $1 \times 10^5$ Mϕ were plated onto 96-well plates and infected with *Leishmania* for 30 min as described above. Extracellular parasites were removed by

washing and reincubated for an additional 1 h in PBS containing 129 mM $CaCl_2$, 109 mM $MgCl_2$, 555 mM D-glucose, 100 U/ml of peroxidase (Sigma) and 0.56 mM of phenol red (Sigma). After 1 h at 37 °C in a 5%-$CO_2$ atmosphere, the absorbance of the supernatants was estimated at $OD_{650}$ in a Molecular Device 96-well microplate reader after mixing with 10 µl of 1 N NaOH per well. $H_2O_2$ was quantified by comparison with a standard curve prepared with known concentrations of $H_2O_2$. Non-infected cells and cells treated with 100 ng/ml phorbol myristate acetate (PMA) served as control.

### 2.6. Determination and inhibition of nitrite accumulation

$NO_2^-$ was determined in culture supernatants. Equal volumes of cell culture medium were mixed with Griess reagents (1% sulfanilamide, 0.1% naphtylethylenedamine, and 2.5% $H_3PO_4$). This mixture was distributed in a 96-well plate and estimated at $OD_{570}$ in a Molecular Device 96-well microplate reader. The standard curve used $NaNO_2$ as a reference, in concentrations from 1 to 200 µM, and the results were expressed in µM per number of cells in culture. To inhibit NO production 100 mM of AMG (aminoguanidine), a competitive inducible NO synthase (iNOS) inhibitor, was added to the cultures at the same time as IFN-γ ± TNF-α.

### 2.7. NO-releasing agents

NO-releasing agent, *S*-nitroso-*N*-acetylpenicillamine (SNAP), was purchased from Calbiochem (La Jolla, CA). SNAP was dissolved in dimethyl sulfoxide (Sigma) at a concentration of 100 mM, stored at –20 °C. Cells were pre-treated every 4 h for 48 h with 100 µM of SNAP in culture medium.

### 2.8. Cytokine treatment of Mϕ

Mϕ adhered to glass coverslips were incubated for 12–18 h with 100 UI/ml of recombinant (r)IFN-γ (Pharmingen, San Diego, CA or R&D system, Minneapolis, MN), in combination or not with 100 UI/ml of r tumor necrosis factor (TNF)-α, kindly provided by Dr. Jeanne Wietzerbin, or 10 ng/ml of r transforming growth factor (TGF)-β (R&D System) and subsequently infected with *L. amazonensis* or *L. major* stationary-phase promastigotes. In parallel, cells were infected and treated with rTNF-α or rTGF-β alone. After 12 h of infection, cells were washed to remove free parasites and fresh cytokines were replaced using similar concentrations. Non-infected Mϕ were used as control.

### 2.9. Neutralization of cytokines produced by infected Mϕ

Mϕ were pre-treated with a combination of 100 UI/ml of rIFN-γ and 10 µg/ml of anti-TNF-α (R&D System) or normal goat serum (Sigma), as control, for 12–18 h. Subsequently, Mϕ were infected with *L. amazonensis* or *L. major* and, at time 0, they were washed to remove non-internalized parasites, and antibodies were replaced at similar concentrations. After 72 h of infection, the percentage of infected cells was estimated.

### 2.10. Cytokine and iNOS mRNA detection

Total mRNA was extracted using Trizol LS reagent. Complementary DNA (cDNA) was synthesized using Superscript II reverse transcriptase (Gibco laboratories) at 42 °C for 55 min. Expression of mRNA was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) for TNF-α, TGF-β, IL-10 and iNOS. PCRs were performed on a PT-100 thermal cycler (Perkin-Elmer) in a reaction containing 2.5 mM PCR nucleotide mix (Roche Molecular Biochemicals), forward and reverse primers (0.5 pmol/µl each), 1× PCR buffer with 1.7 mM $MgCl_2$, 2 µg/µl cDNA and 0.025 U/µl *Taq* polimerase (Roche Molecular Biochemicals) made up to 20–30 µl with distilled water. The same master mix containing all reagents was used for each sample. Reaction conditions were as follows: 94 °C for 5 min, then 35 cycles of denaturation condition at 95 °C for 1 min, annealing at 60 °C (TGF-β, TNF-α) or 58 °C (iNOS) or 55 °C (IL-10) for 2 min, and polymerization at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. PCR products were resolved by agarose gel electrophoresis (1.6% gel) and stained with ethidium bromide.

Primer sequences were as follows: TGF-β sense, 5'-CCAGATCCTGTCCAAACTAAGG-3'; TGF-β antisense, 5'-GAATCGAAAGCCCTGTATTCC-3'; TNF-α sense, 5'-ATGCACCACCATCAAGGACT-3'; TNF-α antisense, 5'-GCAAAAGAGGAGGCAACAAG-3'; IL-10 sense 5'-AGAAAAGAGAGCTCCATCATGC-3'; IL-10 antisense, 5'-AATCACTCTTCACCTGCTCCA-3'; iNOS sense, 5'-GTTCCAGAATCCCTGGACAA-3'; iNOS antisense, 5'-AACATTTCCTGTGCTGTGCTACA-3'; hypoxanthine guanine phosphoribosyl transferase (HPRT) sense, 5'-AGCTTGCTGGTGAAAAGGAC-3'; HPRT anti-sense, 5'-TTATAGTCAAGGGCATATCC-3'.

### 2.11. Statistical analysis

All experiments were done in triplicate and independently repeated at least two times. Most of the results are expressed as mean ± S.E.M. of three or more experiments or, when indicated, one is representative of similar experiments. Statistical significance between experimental groups and controls was analyzed by Student's *t*-test or by one-way ANOVA to compare three or more groups. A $P$ value of $<0.05$ was considered statistically significant.

## 3. Results

### 3.1. L. amazonensis *and* L. major *induce different infection patterns in CBA/J Mϕ*

CBA/J PEC Mϕ were cultivated in complete DMEM medium for at least 24 h at 37 °C and then were infected with *L.*

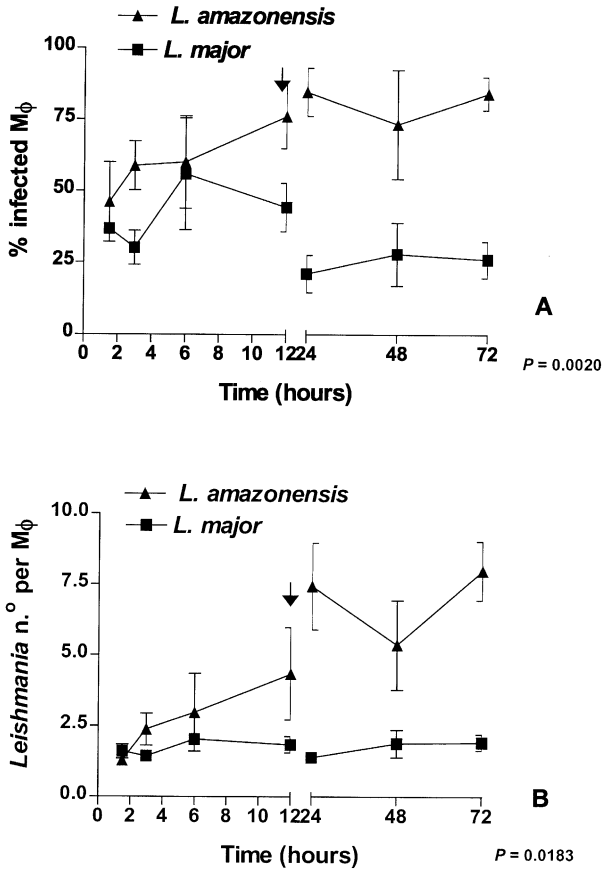


Fig. 1. Kinetics of CBA/J Mϕ infection by *L. amazonensis* or *L. major*. (A) Percentage of *L. amazonensis*- or *L. major*-infected Mϕ; (B) Parasite burden of *L. amazonensis*- or *L. major*-infected Mϕ. PEC Mϕ were infected with stationary-phase promastigotes at a ratio of 10:1. The percentages of infection and parasite number per Mϕ were determined. These cells were fixed at 1.5, 3.0, 6.0 and 12 h after infection. Twelve hours later, the time 0 (arrows), cells were washed and re-incubated for an additional 24, 48 and 72 h. This data represents the mean ± S.E.M. of five experiments; *P* values are indicated in each figure (paired Student's *t*-test).

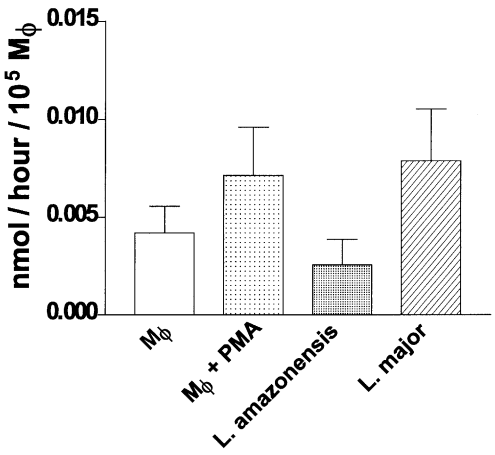


Fig. 2. Release of hydrogen peroxide by *L. amazonensis*- or *L. major*-infected Mϕ. PEC Mϕ were plated and infected with *L. amazonensis* or *L. major* stationary-phase promastigotes at a ratio of 10:1 for 30 min, and extracellular parasites were removed by washing. Non-infected cells and cells treated with 100 ng/ml PMA served as control. The amount of $H_2O_2$ was measured after 1 h incubation at 37 °C according to the peroxide-mediated $H_2O_2$-dependent oxidation as indicated in Material and methods. Results are reported as mean ± S.E.M. of two experiments done in triplicate.

*amazonensis* or *L. major* stationary-phase promastigotes. Kinetic studies were performed to compare the capacity of each parasite to infect CBA/J mouse Mϕ. We observed no differences between the percentages of infected Mϕ or parasite numbers per Mϕ until 6 h after parasite addition to the cultures (Fig. 1A, B). However, after 12 h of infection, we detected significant differences between the two groups. In order to analyze the effect of long-term infection, after 12 h of infection, at time 0, Mϕ were washed to remove non-internalized parasites and were cultivated for an additional 24, 48 and 72 h. After each of these periods, cells were fixed and analyzed according to the parameters described above. At time 0, the percentage of *L. amazonensis*-infected cells was 76.1 ± 11.2% ($n = 3$) and increased to 84.8 ± 5.8% ($n = 8$) 72 h postinfection. On the other hand, the percentage of *L. major*-infected cells decreased from 44.3 ± 8.4 % ($n = 3$) at time 0 to 26.1 ± 6.3% ($n = 8$) after 72 h of infection ($P = 0.002$) (Fig. 1A). Parasite burden was then determined and an enhancement was observed from 3.0 to 8.0 parasites per *L. amazonensis*-infected cell from 6 to 72 h postinfection. On the other hand, 2.0 parasites per *L. major*-infected Mϕ were detected, and there was no variation in this number for all periods analyzed ($P = 0.0183$) (Fig. 1B). Similar results were observed by infecting Mϕ at 35 °C (not shown), indicating that the *L. amazonensis* strain used in our system is adapted to infect cells at 37 °C. Together these results suggest that the first contact between CBA/J Mϕ and *L. major* activates specific host cell responses, which are sufficient to contain parasite infection, in contrast to Mϕ response to *L. amazonensis* infection.

### *3.2.* L. amazonensis *inhibited $H_2O_2$ accumulation in Mϕ cultures*

To study the contribution of radical oxygen intermediates (ROI) in *Leishmania* killing, we compared $H_2O_2$ production by control non-infected, PMA-treated cells and *L. amazonensis*- or *L. major*-infected Mϕ. Fig. 2 shows that *L. amazonensis*-infected Mϕ produced half of the $H_2O_2$ production as that of *L. major*-infected Mϕ, although this difference was not statistically significant. In addition, the microbicidal molecule production by *L. major*-infected cells was similar to that of the positive control cells. This data suggests that the first contact of the parasite with Mϕ induced differential ROI production, and this may be responsible, at least in part, for the differences in CBA/J Mϕ capacity to destroy *L. amazonensis* and *L. major* parasites.

### *3.3. IFN-γ activation of infected Mϕ impaired* L. major *but not* L. amazonensis *infection*

IFN-γ was added to the cultures to evaluate its role in the control of *L. amazonensis* infection. PEC Mϕ were pre-treated with rIFN-γ and then cells were infected with *L.*

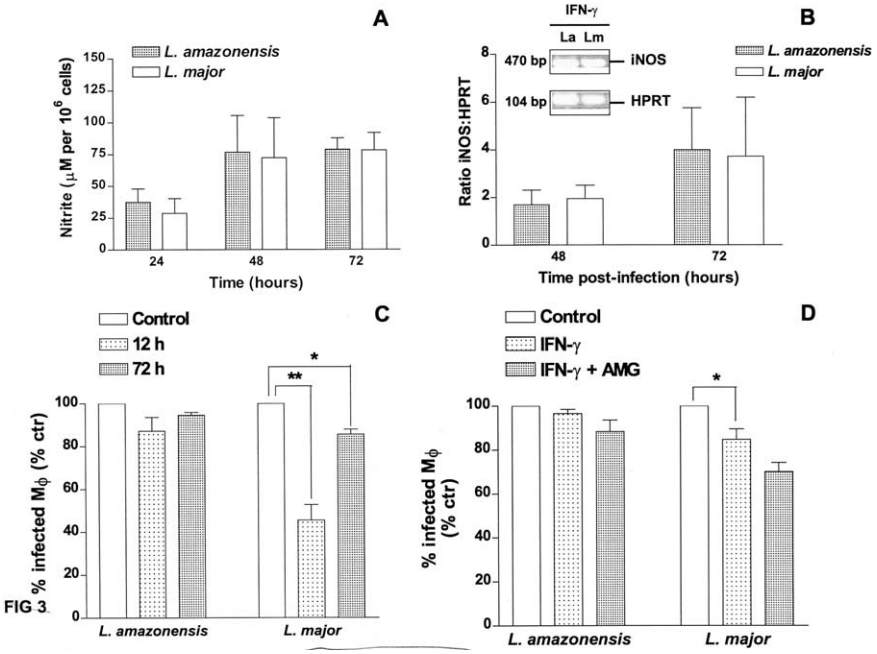


Fig. 3. Effect of IFN-γ in *L. amazonensis*- or *L. major*-infected Mϕ. PEC Mϕ were cultured and infected as described in Fig. 1 and served as control. In parallel, Mϕ were pretreated with IFN-γ (100 UI/ml) ± AMG (1 mM) during 12–18 h. After this time, cells were infected; 12 h later they were washed to remove free parasites and then IFN-γ was replaced using similar concentration. (A) Nitrite levels were measured by Griess reaction in supernatants from cultures of $1 \times 10^6$ cells infected with *L. amazonensis* or *L. major* and treated with IFN-γ. Data are means of three experiments ± S.E. ($P > 0.05$). (B) iNOS and HPRT gene products of specific amplification by RT-PCR. Densitometric analysis of levels of iNOS-specific mRNA were determined by RT-PCR after standardization of cDNAs for HPRT gene expression in IFN-γ-treated Mϕ infected with *L. amazonensis* or *L. major*. ($P > 0.05$, Student's *t*-test). (C) Effect of IFN-γ on the percentage of infection. Control of infection was considered 100% (*L. amazonensis* 12 h = 76.1 ± 11.2%, $n = 3$ and 72 h = 84.3 ± 5.8%, $n = 8$; *L. major* 12 h = 44.3 ± 8.4% and $n = 3$, 72 h = 26.1 ± 6.3%, $n = 8$). (D) Effect of AMG on the percentage of infected cells treated with IFN-γ 72 h postinfection. Control of infection was considered 100% (86.3 ± 3.9% in *L. amazonensis* infection and 30.5 ± 6.1% in *L. major* infection, $n = 8$). In C and D the results represent the percentage of infection related to control cells. Data are means of ($n$) experiments ± S.E. (ANOVA).

*amazonensis* or *L. major* promastigotes. Non-internalized promastigotes were washed out at time 0, and cells were reincubated for an additional 24, 48 and 72 h in the absence or in the presence of IFN-γ. NO was measured in cell supernatants to evaluate Mϕ activation. Non-infected cells and infected cells without IFN-γ pretreatment served as controls, and produced small amounts of NO. As expected, non-infected Mϕ treated with IFN-γ produced high levels of NO after 72 h of infection (47.5 ± 11.3 μM per $10^6$ cells). Surprisingly, there was no difference between NO produced by *L. amazonensis*- and *L. major*-infected PEC Mϕ during the whole period ($P = 0.1931$) (Fig. 3A). To confirm similar NO production by these cells, we evaluated the iNOS mRNA expression by RT-PCR. After 12 h (not shown) until 72 h postinfection, iNOS mRNA expression was detected at a similar level by IFN-γ-pre-stimulated *L. amazonensis*- and *L. major*-infected Mϕ (Fig. 3B).

Although similar amounts of NO were induced by IFN-γ in *L. amazonensis*- and *L. major*- infected cells (Fig. 3A), IFN-γ induced a not very significant reduction in *L. amazonensis* infection, related to control cells at time 0 and 72 h postinfection (Fig. 3C). In contrast, IFN-γ significantly reduced the percentage of *L. major* infection at time 0 ($P < 0.001$) (Fig. 3C). After 72 h of infection, the IFN-γ effect on the percentage of *L. major* infection was smaller than at time 0, but was still significant ($P < 0.05$) (Fig. 3C). This data indicates that, in contrast to *L. major*, *L. amazonensis* is resistant to IFN-γ-induced NO production by CBA/J Mϕ.

To evaluate the role of NO in our system, AMG was added to the cultures. AMG completely blocked NO production induced by IFN-γ in infected cells (not shown). Surprisingly, its addition to CBA/J Mϕ neither modified *L. amazonensis* infection ($P > 0.05$) nor reversed IFN-γ inhibition of *L. major*-infected cells ($P > 0.05$) (Fig. 3D). The NO donor SNAP was then added to the cultures to test whether very high NO concentrations have any effect on the control of *Leishmania* infection. Indeed, NO concentration detected in SNAP-treated cells was higher than in IFN-γ-treated Mϕ, and the percentage of infection was reduced in both *L. amazonensis*-and in *L. major*-infected Mϕ compared to levels observed in non-treated infected cells (Fig. 4A, B).

### *3.4. Role of TNF-α in* Leishmania *infection*

As the role of TNF-α in *L. amazonensis* infection has not been evaluated until now, and the control of *L. major* infection is still controversial [29–31], we tested whether IFN-γ-activated Mϕ inhibited *Leishmania* infection by a mechanism dependent on TNF-α. We first determined TNF-α mRNA levels at periods before and after time 0 and we were only able to detect expression in IFN-γ-activated cells at time 0. In all experiments ($n = 5$), TNF-α mRNA levels in IFN-γ-

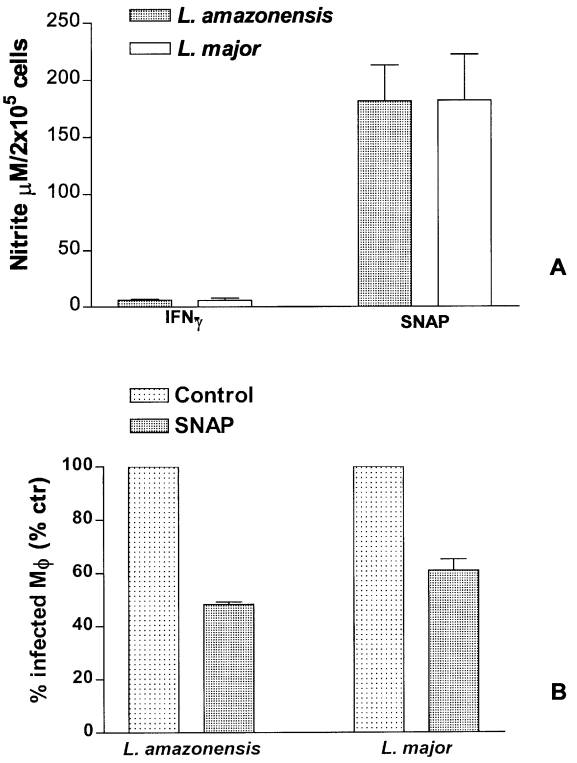


Fig. 4. High NO concentrations inhibit Mϕ infection by *L. amazonensis* and *L. major*. (A) Nitrite levels after SNAP addition. (B) Percentage of Mϕ infection with *L. amazonensis* or *L. major* in SNAP-treated cells. Control of infection was considered 100% (*L. amazonensis* 73.10 ± 8.8 and *L. major* 33.37 ± 6.7). PEC Mϕ were cultured and infected as described in Fig. 1 and served as control. Cells were infected and 12 h later were washed to remove free parasites. After this time cells were re-incubated and treated every 4 h for 48 h with 100 μM of SNAP. Results are reported as mean ± S.E.M. of two experiments done in triplicate, $P < 0.0001$ (ANOVA).

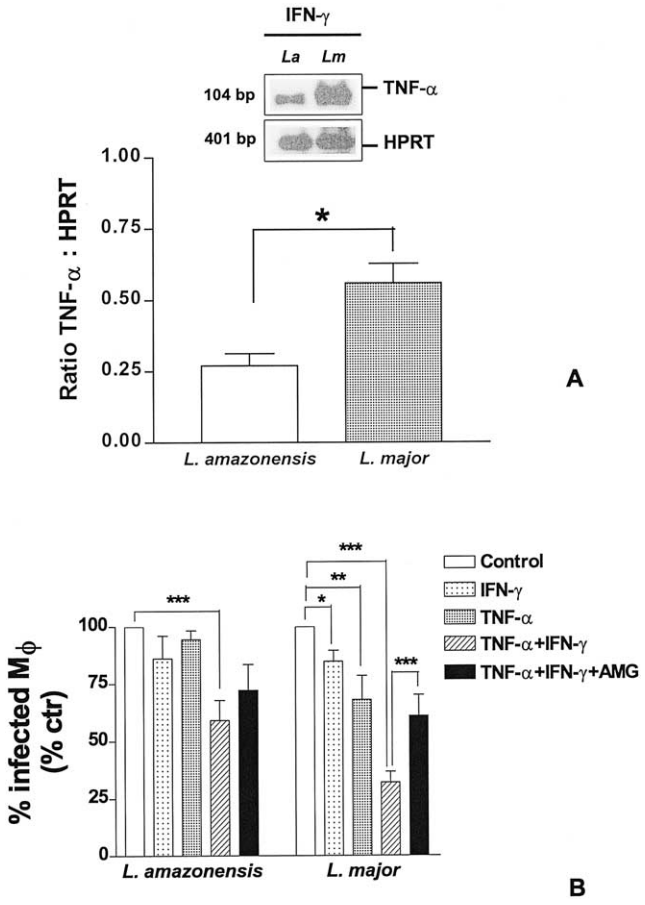


Fig. 5. Role of TNF-α in Mϕ infection by *Leishmania*. (A) TNF-α and HPRT gene products of specific amplification by RT-PCR 12 h postinfection. Densitometric analysis of levels of TNF-α-specific mRNA were determined by RT-PCR after standardization of cDNAs for HPRT gene expression in IFN-γ-treated Mϕ infected with *L. amazonensis* or *L. major*. This data represent the means of five experiments ± S.E. (B) AMG effect on the percentage of infected cells treated with TNF-α plus IFN-γ 72 h postinfection. Control of infection was considered 100% (*L. amazonensis* = 83.6 ± 3.8% $n = 12$; *L. major* = 30.5 ± 6.1%, $n = 12$). Cells were cultured as described in Fig. 1 and pretreated with IFN-γ (100 UI/ml) ($n = 9$) or IFN-γ (100 UI/ml) plus TNF-α (100 UI/ml) ($n = 6$) ± AMG (1 mM) ($n = 3$) during 12–18 h, after 12 h MΦ were washed to remove free parasites and then cytokines plus AMG were replaced using similar concentrations. This data represents the means of experiments ($n$) ± S.E. Significant differences are indicated in the figure, $P < 0.05$ *, $P < 0.01$** and $P < 0.001$ or $P < 0.0001$ *** (ANOVA).

activated *L. amazonensis*-infected Mϕ were half of those expressed by *L. major*-infected cells ($P = 0.022$) (Fig. 5A). As we were only able to detect a very low expression in *L. amazonensis*-infected cells, anti-TNF-α was only added to IFN-γ-activated *L. major*-infected cells. Anti-TNF-α was able to completely reverse the reduction in *L. major* infection induced by IFN-γ (data not shown). Throughout the time postinfection we were not able to detect TNF-α in cell supernatants.

The effect of TNF-α added exogenously was then tested. Recombinant TNF-α was added to the cultures alone or at the same time as rIFN-γ. As expected, after 72 h of infection, rTNF-α alone significantly reduced the percentage of *L. major*-infected cells (Fig. 5B), and associated with rIFN-γ was able to significantly reduce the percentage of both *L. amazonensis*- ($n = 5$; $P < 0.001$) and *L. major*-infected cells ($n = 5$; $P < 0.001$). As we have observed in IFN-γ-treated cells, NO production was similar in *L. amazonensis*- or *L. major*-infected cells treated with both IFN-γ and TNF-α. Despite the synergic effect on NO production by TNF-α added to IFN-γ-treated cells, three times more than in IFN-γ-treated cells, AMG did not reverse the IFN-γ plus TNF-α effect on *L. amazonensis* infection ($n = 3$; $P = 0.504$). Instead, the iNOS inhibitor partially reversed the IFN-γ plus TNF-α inhibition of *L. major* infection ($n = 3$, $P = 0.037$) (Fig. 5B).

### *3.5. Effect of TGF-β and IL-10 on* L. amazonensis- *or* L. major-*infected Mϕ*

We also investigated the role of TGF-β and IL-10, Mϕ-produced cytokines implicated in driving the Th-2 response. We first compared mRNA levels of expressed cytokines by *L. amazonensis*- or *L. major*-infected cells, then measured cytokines in infected cell supernatants. TGF-β mRNA expression increased according to time of infection (not shown), reaching its maximum at 72 h postinfection. As shown in Fig. 6A, after infection, TGF-β mRNA expression is slightly

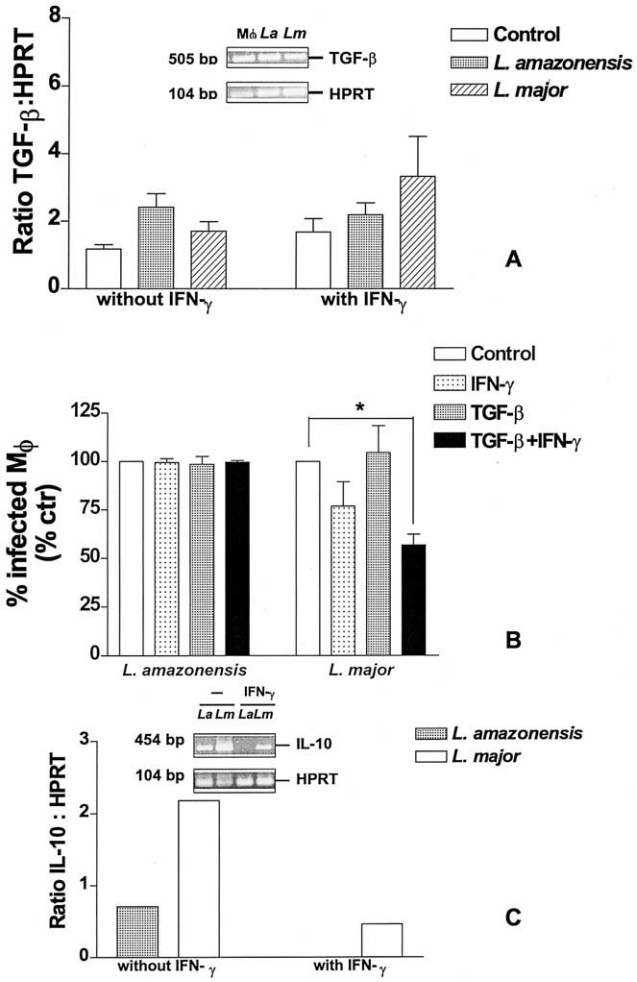


Fig. 6. Expression of cytokines and TGF-β effect on *L. major*- or *L. amazonensis*-infected Mϕ. (A) TGF-β and HPRT gene products of specific amplification by RT-PCR 72 h postinfection. Densitometric analysis of levels of TGF-β-specific mRNA were determined by RT-PCR after standardization of cDNAs for HPRT gene. PEC Mϕ ($1 \times 10^6$per ml) were cultured and infected as indicated in Fig. 3. This data represents the means of five experiments ± S.E. ($P > 0.05$, one-way ANOVA). (B) Effect of TGF-β ± IFN on *L. amazonensis* or *L. major* infection at 72 h. Cells were cultured as described in Fig. 1 and Mϕ were pre-treated with IFN-γ (100 UI/ml) ± TGF-β (10 ng/ml) during 12–18 h. After 12 h of infection, cells were washed to remove free parasites and cytokines were replaced using similar concentration. Control of infection was considered 100% (*L. amazonensis* = 93.8 ± 1.7%, $n = 3$, $P = 0.9094$; *L. major* 35.8 ± 8.6%, $n = 3$, $P = 0.0149$, ANOVA). Results represent the percentage of infection related to control cells. (C) IL-10 and HPRT gene products of specific amplification by RT-PCR 72 h post infection. Densitometric analysis of levels of IL-10-specific mRNA was determined by RT-PCR after standardization of cDNAs for HPRT gene expression. PEC Mϕ ($1 \times 10^6$ per ml) were cultured and infected as indicated in Fig. 3. This data is one representative of two similar experiments ± S.E.

higher in the remaining cells ($P = 0.4061$) and upon IFN-γ activation ($P = 0.7352$) than in control non-infected Mϕ. Similarly, levels of TGF-β in supernatants of *L. amazonensis*- and *L. major*-infected cultures showed no differences, and were slightly higher than in control non-infected cells (not shown). In the next step, cells were treated before and during infection with only rTGF-β, or both rIFN-γ and rTGF-β. Addition of rTGF-β did not modify either *L. amazonensis* or *L. major* infection. As shown in Fig. 6B, rTGF-β in combination with rIFN-γ significantly reduced the percentage of *L. major* infection in CBA/J Mϕ, although it did not modify the percentage of *L. amazonensis*-infected cells.

Since we did not detect IL-10 in cell supernatants, we evaluated the IL-10 mRNA expression by infected cells. We were only able to detect IL-10 mRNA expression at 72 h postinfection. Non-stimulated *L. major*-infected cells expressed two-fold higher IL-10 mRNA as *L. amazonensis*-infected cultures. IFN-γ treatment was able to completely down-regulate IL-10 mRNA expression in *L. amazonensis* and reduced the expression in *L. major*-infected cultures by 4.7-fold (Fig. 6C).

## 4. Discussion

In the present report, we used an in vitro assay to study the interaction between *L. amazonensis* or *L. major* promastigotes and inflammatory CBA/J mouse Mϕ. Our goal was to mimic a very early event in the interaction between *Leishmania* and the host cell. We showed for the first time that two distinct *Leishmania* species trigger different responses to the infection in the same Mϕ, even before Mϕ have been activated by rIFN-γ. Previously, we demonstrated that CBA/J mice infected with *L. amazonensis* showed dissemination of parasites, and the animals did not survive, whereas CBA/J infected with *L. major* resulted in a pattern of resistance and cure [7].

A possible bias in our system is the use of stationary-phase promastigotes instead of purified metacyclic forms. However, the limitations of this are minimized by the fact that we are comparing the infection of the same Mϕ in two different species of *Leishmania*, and the percentage of complement-resistant promastigotes was similar in *L. amazonensis* and *L. major* stationary-phase promastigotes.

We showed that *L. amazonensis*-infected Mϕ generated half the $H_2O_2$ of that produced by *L. major*-infected Mϕ. Our data suggest that the incapacity of CBA/J Mϕ to destroy *L. amazonensis* depends, at least in part, on the deficiency of ROI production, emphasizing the role of innate immune elements on the determination of *Leishmania* infection outcome. Although we did not address this question, it has been demonstrated that *Leishmania*'s ability to survive inside cells may depend on the interaction of parasite surface molecules with Mϕ receptors [32–35]. Recently, it was demonstrated that one mechanism used by *L. amazonensis* to survive in the host cell is the very early acquisition of amastigote features inside the parasitophorous vacuole [36]. This interaction might influence activation of host signaling pathways and parasite intracellular fate inside CBA/J Mϕ.

Twelve hours postinfection, in IFN-γ activated Mϕ, TNF-α expression was lower in *L. amazonensis*- than in *L. major*-infected Mϕ. In addition, neutralization of TNF-α led to a higher parasite burden in *L. major*-infected Mϕ pretreated with IFN-γ, indicating that TNF-α expression lead to

a reduced percentage of *L. major* infection. TNF-α only plays a role in *L. major* killing in IFN-γ pre-treated cells, as anti-TNF-α did not reverse *L. major* killing in non-activated infected Mϕ (data not shown). This data indicates that TNF-α induced by IFN-γ in *L. major*-infected cells was sufficient to enhance Mϕ killing mechanisms. Similarly to our study, it has been demonstrated that rIFN-γ by itself can activate peritoneal resident or starch-elicited Mϕ to destroy intracellular *L. major* by a mechanism dependent on TNF-α induction [18,31,37]. However, in most of the studies, it was demonstrated that IFN-γ activates Mϕ to kill *L. major* only if associated with TNF-α or LPS [17,20,38,39].

We also demonstrated that the addition of TNF-α to IFN-γ-activated Mϕ markedly enhanced Mϕ leishmanicidal activity, which confirms the data found in the literature, suggesting that TNF-α may induce a second signal to IFN-γ-treated Mϕ [40,41], contributing to the activation of Mϕ killing of intracellular parasites. Our data also indicates that, although *L. amazonensis* is more resistant to innate and IFN-γ-induced killing mechanisms than *L. major*, parasites can be destroyed when cells were activated by both IFN-γ plus TNF-α.

In CBA/J Mϕ the effect of IFN-γ alone against *Leishmania* was not dependent on NO, since AMG addition did not modify *L. amazonensis* or *L. major* infection. In addition, TNF-α- plus IFN-γ-treated cells produced three times more NO than the cells treated with IFN-γ alone and addition of AMG, which completely blocked NO production, did not modify *L. amazonensis* infection and partially reversed the inhibition in *L. major* infection. These data indicate that the effect of the two cytokines against *Leishmania* is only partially dependent on NO. Previously, we have shown that in vivo establishment and dissemination of *L. amazonensis* infection are not related to an inability of Mϕ to produce NO [7], but that, in comparison to *L. major*, *L. amazonensis* is more resistant to being killed by the levels of NO or other host killing mechanisms produced in the early phases of the infection. These data is in accordance with another study where it was demonstrated that control of *L. amazonensis* infection by inflammatory mediators such as platelet activating factor is independent of NO [42]. Recently, it was demonstrated that in vivo as well as in vitro TNF-α induces *L. major* killing by a mechanism partially independent on NO. TNF-α knockout C57BL/6 mice cannot control *L. major* infection, although iNOS protein is readily detectable in skin lesions and draining LNs [30]. Furthermore, we showed that SNAP, a chemical NO donor, added to CBA/J Mϕ highly enhanced NO in the cultures, but did not sterilize infected Mϕ. Together, this data support the idea that NO can kill intracellular parasites, but its effect is potentially enhanced, depending on whether or not NO is associated with other host toxic molecules, such as oxygen reactives [19,43], similarly to the model of in vitro control of *Salmonella typhimurium* infection [44].

In CBA/J Mϕ expressed TGF-β mRNA and TGF-β in cell supernatants were produced in the same levels by *L. amazonensis*- and *L. major*-infected cells, slightly higher than in non-infected cells. In addition, we showed that TGF-β alone had no effect on parasite infection, but the addition of this cytokine in the presence of IFN-γ significantly reduced the percentage of *L. major*-infected cells, demonstrating that in vitro TGF-β potentialized the IFN-γ effect on CBA/J Mϕ activation to destroy *L. major*. In contrast to our work, data found in the literature [45,46] have clearly demonstrated that in vivo TGF-β suppresses resistance to *L. major* infection, and that treatment with TGF-β promotes disease in resistant C57BL/6 mice infected with either *L. amazonensis* or *L. braziliensis*. On the other hand, treatment of susceptible BALB/c mice with anti-TGF-β antibody enhanced resistance to *L. amazonensis* infection [46]. It has been shown that TGF-β influences CD4+ T-cell maturation, although contradictory effects on T-cell differentiation have been reported in various in vitro studies showing that TGF-β can either promote or suppress Th-1 development [47–50].

IL-10 mRNA expression was higher in non-stimulated infected cells than in IFN-γ-treated Mϕ. This result is in accordance with data in the literature, which demonstrates the IFN-γ role on modulation of IL-10 production [51]. Furthermore, we showed that *L. major* induced 3-fold higher expression of IL-10 mRNA than *L. amazonensis*-infected Mϕ. In our previous work we demonstrated a 4-fold higher production of IL-10 by LN cells of *L. major*-infected mice over those produced by LN cells of *L. amazonensis*-infected animals [7]. These data support the idea that in CBA/J mice, IL-10 can exert a dual role in the immune response against *Leishmania*. In a context of Th-2 response as observed in *L. amazonensis*-infected CBA/J mice, IL-10 can exacerbate the infection. On the other hand, IL-10 can possibly modulate a Th-1 type of immune response in *L. major*-infected CBA/J mice.

In summary, we showed that in vitro *L. amazonensis* inactivates and is resistant to innate and IFN-γ-induced Mϕ killing mechanisms. We also demonstrated that TNF-α induced in response to IFN-γ activation is involved in the control of *L. major* infection by a mechanism partially dependent on NO, suggesting that the NO effect is associated with the effect of other host toxic molecules. TGF-β and IL-10 are seemingly not determinants of the infection of CBA/J mouse Mϕ by *L. amazonensis* or *L. major*. In conclusion, the results presented here emphasize the role of the parasites in determining the outcome of *Leishmania* infection, and indicate that the first contact of parasites with Mϕ can translate information, regarding the nature of the pathogen, into differences in produced microbicidal molecules and cytokines by host cells, which play a role in the polarization of the acquired immune response.

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