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Research Article

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RESEARCH ARTICLE

Leishmania (Viannia) braziliensis amastigotes from patients with mucosal leishmaniasis have increased ability to disseminate and are controlled by nitric oxide at the early stage of murine infection

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One sentence summary: Parasites that cause mucosal leishmaniasis are different from parasites that cause only cutaneous lesions.

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ABSTRACT

Mucosal leishmaniasis (ML) caused by *Leishmania (Vianna) braziliensis* usually appears after the healing of the primary lesion when amastigotes disseminate from the infection site to the mucosal area. Here, we investigated murine infection with amastigotes obtained from patients with ML or localized cutaneous leishmaniasis (LCL). Amastigotes were used to infect wild type, IFN-γ KO and inducible nitric oxide synthase (iNOS) KO mice. Amastigotes from patients with LCL induced lesions that appeared earlier in IFN-γ KO than parasites from ML. The lesion after infection with ML appeared early in iNOS KO than in IFN-γ KO mice and in iNOS KO mice parasites from ML and LCL cause similar lesions at the initial phase of infection, while parasites from ML induced greater lesions than the ones from LCL at the late phase. A greater number of parasites were observed in spleen of IFN-γ KO and iNOS KO mice infected with amastigotes from patients with ML than those with LCL. Parasites from ML infect a lower percentage of macrophages and are killed independent on IFN-γ and dependent on NO. The data suggest that amastigotes responsible for mucosal lesion in humans develop slowly on the initial phase of infection due to high susceptibility to NO and they have an increased ability to disseminate.

Keywords: *Leishmania (V.) braziliensis*; amastigote; nitric oxide; mucosal leishmaniasis; Interferon-gamma

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INTRODUCTION

Human tegumentary leishmaniasis is a complex disease with a broad clinical spectrum. Among the different forms, the localized cutaneous leishmaniasis (LCL) caused by *Leishmania (Viannia) braziliensis* is less severe. Yet, the same parasite is able to cause the mucosal leishmaniasis (ML) form that evolves into a progressive and destructive disease causing respiratory dysfunction and serious social and psychological problems to patients (Marsden 1986; Costa *et al.* 1987; Lessa *et al.* 2007).

Metacyclic promastigotes are the parasite's forms transmitted by insect vector to the vertebrate host. This form is internalized by phagocytes in the first hours after infection; they rapidly differentiate into intracellular amastigotes that undergo several rounds of binary division, and are eventually released to be internalized by other neighboring cells repeating the cycles of proliferation and intracellular infection (Van Assche *et al.* 2011). Most patients develop the LCL form with a single ulcerated skin lesion that develops at the infection site which, in some cases, may heal spontaneously. In contrast, the ML form appears after the apparent healing of the primary lesion. Patients develop one or more non-healing invasive satellite lesions and the disease is often difficult to treat (Marsden 1986; Acestor *et al.* 2006)

The determining causes of the evolution towards ML are not clear, but both host and parasite factors must be implicated (Campos *et al.* 2008; Ramasawmy *et al.* 2010; Maretti-Mira *et al.* 2012; Alves-Ferreira *et al.* 2015). In LCL the parasites are confined to the skin and lymphoid organs in the vicinity of the lesion (Nylen and Eidsmo 2012), whereas in ML the parasites disseminate beyond the infection site causing one or more metastatic mucosal lesions (Marsden 1994; Acestor *et al.* 2006).

The control of *L. (V.) braziliensis* parasites in vertebrate host depends on the development of a Th1-type immune response. Th1 cells are characterized by producing high levels of interferon-γ (IFN-γ), a crucial cytokine to activate macrophages to kill the parasite (Silveira *et al.* 2009; Schnorr *et al.* 2012). Once activated by IFN-γ, macrophages increase the production of inducible nitric oxide synthase (iNOS) (Liew and O'Donnell 1993; Murray *et al.* 2014). The iNOS uses L-arginine to produce nitric oxide (NO), one of the most potent leishmanicidal agents produced by macrophages (Liew and O'Donnell 1993; Rocha *et al.* 2007).

The fact that ML often appears after the healing of the initial primary lesion suggests that the host immune response is capable of exerting control on the proliferation of amastigotes at the site of infection. Nevertheless, in some hosts, the appearance of the metastatic lesions in ML indicates that amastigotes disseminated from the initial infection site to the mucosa in spite of the immune response.

Because amastigote is the parasite form present in the vertebrate host when primary lesion is healing, whereas promastigotes are present only in the initial phase of infection, the question arose whether amastigotes obtained from patients with ML would have different biological capabilities than parasites derived from patients with LCL.

We undertook this study to search for possible differences in the murine infection when using amastigotes derived from patients with LCL or ML. As infections caused by *L. (V.) braziliensis* in most wild-type (WT) murine models are well controlled, mice deficient in IFN-γ or iNOS were used to improve the ability to detect such differences. The course of infection in the infected paw and parasite loads were compared in mice infected with each of these amastigote populations.

MATERIALS AND METHODS

Mice

Male or female C57BL/6 mice with disrupted IFN-γ genes (IFN-γ KO mice) were used to obtain amastigotes from the infected footpad after inoculation with the human lesion isolates. For experiments, female WT C57BL/6, IFN-γ KO and iNOS KO mice were used, aged 8–10 weeks. iNOS and IFN-γ KO mice were originally purchased from Jackson Laboratories, ME/USA. The mice were bred at the animal facilities of Federal University of Goiás/IPTSP, Brazil and maintained in a clean conventional mouse facility with *ad libitum* access to water and food. All the experimental procedures were conducted according to the guidelines of the Animal Research Ethical Committee (CETEA) of the Federal University of Goiás, approved under the protocol 062/2010.

Parasites and infection

In this study, we used amastigotes obtained from patients with ML (MHOM/BR/2009/ASL9m, MHOM/ BR/2006/PPS6m and MHOM/BR/2008/JBC8m) and LCL (MHOM/BR/2005/RPL5c, MHOM/BR/2008/JCJ8c and MHOM/BR/2007/CSA7c) that were previously characterized by our group (Oliveira *et al.* 2010; Gomes *et al.* 2014). Amastigotes from patients' lesions were collected before any treatment, injected into the footpads of IFN-γ KO mice and recovered from the footpads by finely mincing the tissue. Amastigotes were separated from debris by centrifuging through a 40% and 90% Percoll gradient (GE Healthcare, São Paulo, Brazil) as described before (Gomes *et al.* 2014). Infection of other IFN-γ KO mice with amastigotes was performed to keep the parasites in amastigote form for the experiments.

Groups of three mice were injected in the left footpad with 1000 or 10 000 amastigotes as described in the legends of the figures. Paw swelling was measured weekly using a dial thickness gauge (Mitutoyo, Kawasaki, Japan); the increase in paw thickness was calculated as the difference between the left paw (infected) and the right one (uninfected control paw) measurements.

Estimation of parasitic load

Parasite burden was determined by performing a limiting dilution analysis as previously described (Lima, Bleyenberg and Titus 1997). In brief, the homogenates of infected lesions, draining lymph nodes or spleens were serially diluted (1:10) in 96 well plates (TPP, Techno Plastic Products, Trasadingen, Switzerland) in Grace's Insect Medium supplemented with 20% fetal bovine serum (FBS, Cripion, São Paulo, Brazil), 0.2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO), 100 U mL^{-1} penicillin (Sigma-Aldrich), 100 μg mL^{-1} streptomycin (Sigma-Aldrich) and cultured at 26°C. The plates were observed from 5 to 15 days later for promastigotes' growth. Parasite burden was expressed as the $\log_{10}$ of the reciprocal of the last dilution at which parasites were still observed.

Histopathological analysis

To analyze the local inflammation and fibrosis, the paws of *L. braziliensis*-infected mice were collected and fixed in 10% formalin. Decalcification was performed by immersion of the paws in 5% nitric acid plus 5% paraformaldehyde (Merck, Darmstadt, Germany) in distilled water for 3 days. The material was then

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dehydrated, embedded in paraffin and cut in 5 μm tissue sections which were stained with hematoxylin-eosin.

The sections were analyzed under light microscopy (40× magnification). One hundred microscope fields per section were scored according to the area with inflammatory lesions in relation to the field area. The pathologic processes (necrosis, polymorphonuclear infiltrate, mononuclear infiltrate, hyperemia, angiogenesis, fibrosis, infected cells, parasites, fibroblasts), parasites and infected cells were blindly scored by the observer and classified semi-quantitatively as absent (free of lesions = 0); discreet (spanning 1%–25% of the field area = +); moderate (26%–50% = ++); accentuated (more than 50% = +++). Data of semi-quantitative analysis are presented in Table S1 (Supporting Information).

Infection of bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDM) were obtained as described before (Oliveira *et al.* 2003). Single cell suspensions of bone marrow cells were obtained by flushing the femurs and tibia of mice. The cell suspension (including erythrocytes) was cultured in 6-well culture dishes at 4×10^6 cells mL^{-1}, in RPMI medium (Sigma-Aldrich) supplemented with 2 mM glutamine, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin sulfate, 50 μM 2-ME and 10% FBS. The medium was supplemented with 30% (v/v) L929 (ATCC) cell culture supernatant (sL929-medium). The supernatants were aspirated and medium was replenished every 2 days in order to renew the cytokine and nutrients' source. BMDM were collected after 10 days and cultured at 5×10^5 mL^{-1} at 37°C, 5% CO_2 in 16-well chamber slide (Lab-TeK, Nalge Nunc, Naperville, IL) in complete RPMI medium with or without 50 ng mL^{-1} IFN-γ (R&D Systems, Minneapolis, MN, USA). After 4 h, 5 $\times 10^6$ amastigotes mL^{-1} and 1 μg mL^{-1} lipopolysaccharide (LPS; *Escherichia coli* O111:B4; Sigma-Aldrich) were added to the cultures for 1 h. Cells were washed with phosphate-buffered saline (PBS) to remove non-internalized parasites and slides were collected to infectivity evaluation under a light microscope. In another wells, fresh medium with or without IFN-γ and LPS was added to the cultures and infectivity was evaluated after 72 h. In some experiments, 2,5 mM of N-nitro-L-arginina (L-NAME, Sigma-Aldrich) was added to inhibit NO production.

Determination of nitric oxide (NO) production

NO production was estimated by determining the concentration of nitrite present in the 48 h-culture supernatant of 5×10^5 BMDM mL^{-1} stimulated or not with 50 ng mL^{-1} IFN-γ and 1 μg mL^{-1} LPS in presence or absence of 5×10^6 amastigotes mL^{-1}, using the Griess method (Green *et al.* 1990). This method consists of a colorimetric reaction in which the culture supernatant (50 μL) is incubated with an equal volume of the Griess reagent (1.0% sulfanilamide, 0.1% naphazoline hydrochloride and 2.5% ortho-phosphoric acid) for 10 min at room temperature. The absorbance at 550 nm was determined in an ELISA reader (Multiskan, Thermo Labsystems, Finland). The results were expressed as μM of nitrite based on a standard curve established by known concentrations of sodium nitrite ($NaNO_2$, Sigma-Aldrich) dissolved in culture medium.

In vitro susceptibility of amastigotes to NO

Amastigotes at 5×10^6 parasites 100 μL^{-1} were cultured at 32°C in Grace's Insect Medium (Sigma-Aldrich) supplemented with 2 mM glutamine, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin sulfate and 20% FBS in presence or absence of 25 mM or 100 mM of sodium nitroprussiate (SNP; Sigma-Aldrich), as NO donor. After 2 h, parasites were washed three times with PBS, transferred to a 96-well plate and incubated with 20 μL of 5 mg mL^{-1} MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide; Sigma-Aldrich) in supplemented Grace's medium at 32°C for 20 h. The reaction was stopped by the addition of 100 μL of aqueous solution containing 50% dimethyl sulfoxide and 10% sodium dodecyl sulfate to each well. The plate was incubated at 32°C for 20 h, and the absorbance at 595 nm was determined in an ELISA reader. Assays were performed in triplicate and results expressed as the mean percentage reduction of parasite numbers compared with untreated control wells calculated for five independent experiments.

Statistical analysis

The data are presented as mean ± SD and were compared for significance by Student's *t* test or ANOVA followed by Bonferroni's test using the Graph-Pad Prism Software 5.0 (Inc. San Diego, CA, USA). $P < 0.05$ was considered significant.

RESULTS

The ability of amastigotes to induce lesions in mice was compared by infecting three strains of mice. WT, IFN-γ KO and iNOS KO mice were infected with low (1000) or high (100 000) doses of parasites originally obtained from patients with LCL or ML. WT mice developed small but significant lesions that peaked around 3–4 weeks ($P < 0.05$).There were no significant differences in the infection course of infection caused by different amastigotes in this mouse strain during 8 weeks (Fig. 1A and B).

Lesions induced by two *Leishmania* parasites isolated from patients with LCL (JCJ8c and RPL5c) appeared earlier than lesions triggered by parasites from patients with ML (PPS6m, JBC8m and ASL9m) in IFN-γ KO mice challenged with low dose of amastigote (Fig. 1 C; Fig. S1, Supporting Information). On low dose, JCJ8c and RPL5c caused lesions larger than 1.0 mm after 4 weeks while parasites from patients with ML caused lesions that reached 1.0 mm only after 7 weeks. It is noteworthy that the parasite CSA7c, obtained from patients with LCL, induced a lesion profile similar to the parasites obtained from patients with ML. The infection of IFN-γ KO mice with PPS6m parasite caused the most delayed lesion, which was lower than 1.0 mm on the eighth week. Similar results were observed when high doses were used (Fig. 1D).

All parasites triggered lesions similar and larger than 1.0 mm on the sixth or fourth week, when the dose of 1×10^3 and 1×10^5 were respectively used to challenge iNOS KO mice (Fig. 1E and F and Fig. S2, Supporting Information). It is noteworthy that parasites from patients with ML induced bigger lesions than parasites from patients with LCL in iNOS KO mice after 7 weeks, when a low dose of parasites was used (Fig. 1E).

To evaluate whether the lesion size was related to the parasite load in the infected site, infected mice were euthanized when the average of the group's lesion sizes reached 2.0–3.0 mm. When the lesions did not reach this size, the eighth week was defined as the deadline for euthanasia. WT mice were euthanized on the eighth week, when lesions have already decreased. The number of parasites in the paw, lymph node and spleen of the WT mouse was similar when infections with all parasites were compared (Fig. 2A–F).

IFN-γ KO mice were euthanized between the fifth and eighth week, when there were no significant differences among the lesion sizes (Fig. S1, Supporting Information), except for the mice

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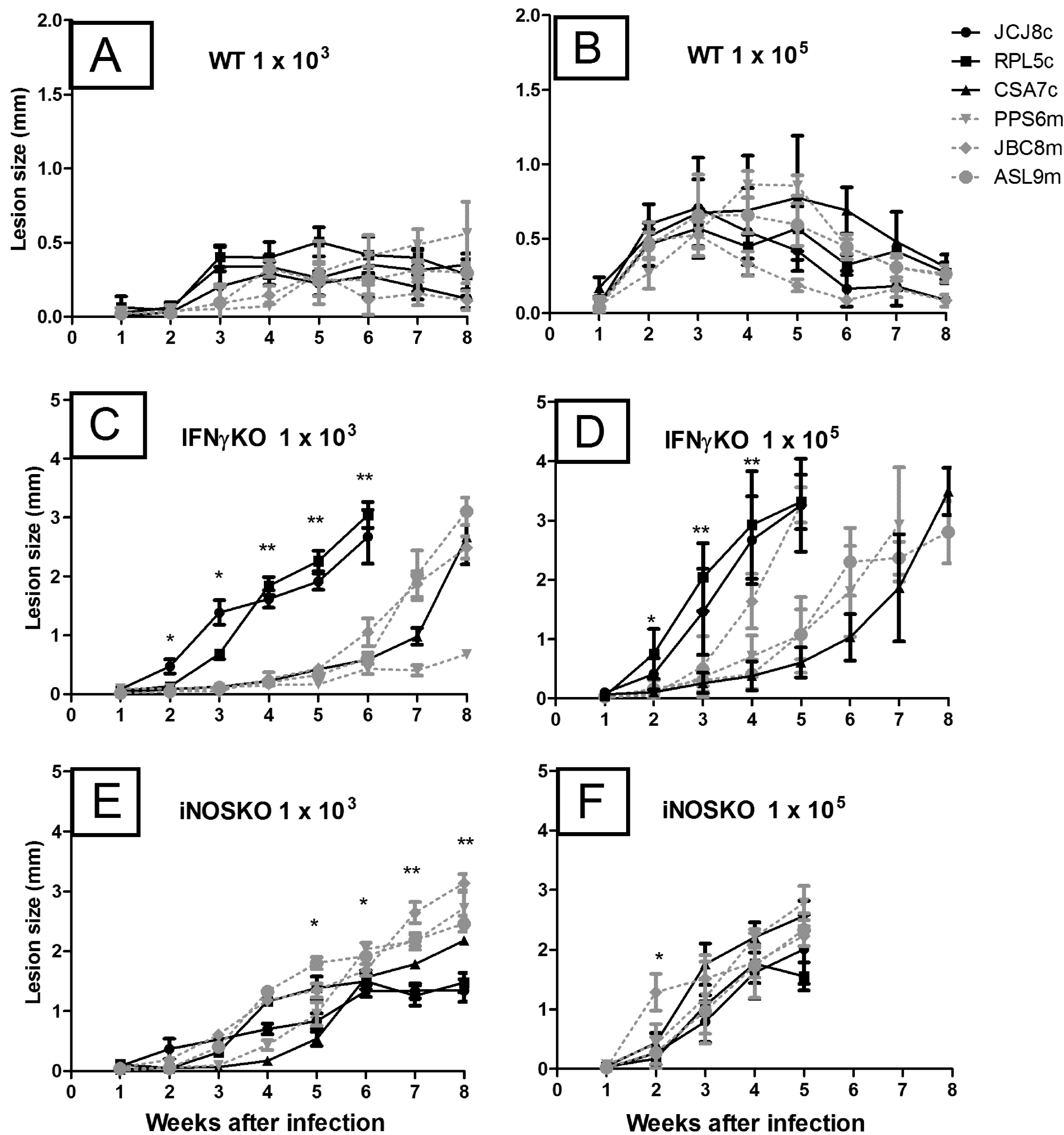

Figure 1. Lesion size triggered in WT, IFN-γ KO and iNOS KO mice by different doses of amastigote. Paw swelling of WT (**A** and **B**), IFN-γ KO (**C** and **D**) or iNOS KO mice (**E** and **F**) was followed weekly after infection with 1000 (A, C and E) or 100 000 (B, D and F) amastigotes of *L. (V) braziliensis* isolated from patients with LCL (JCJ8c, RPL5c or CSA7c, black symbols) or ML (PPS6m, JBC8m or ASL9m, gray symbols). The values presented are the mean ± SD of the difference between the infected and contralateral paw ($n = 12$ mice; four independent experiments with three mice each). Asterisks indicate difference among one or double asterisks two isolates from LCL and the three isolates from ML by ANOVA followed by Bonferroni ($P < 0.05$).

infected with the PPS6m parasite, which had the smallest lesion. When the paw lesions of IFN-γ KO mice infected with parasites from patients with LCL or ML reached the same size, the parasite burden in the infected paw and lymph node was similar, regardless the difference in the time of euthanasia or the dose of parasites used (Fig. 2G–L). Nevertheless, more parasites were observed in the spleen of mice infected with amastigotes from patients with ML than those with LCL.

iNOS KO mice were euthanized at the sixth week, when no significant differences were observed among the lesion sizes, except for the PPS6m-infected mice that developed the largest lesion (Fig. S2, Supporting Information). The number of parasites in the infected paw was similar for all parasite isolates (Fig. 2M and P). A higher parasite burden in iNOS KO mice infected with amastigotes from patients with ML than those with LCL was observed in lymph node and spleen (Fig. 2N–R).

To evaluate whether the differences observed in the course of infection were due to differences in the rate of phagocytosis (1 h) or of killing (72 h) of parasites by macrophages, BMDM from WT and iNOS KO mice were infected with amastigotes from JCJ8c (from patients with LCL) and PPS6m (from patients with ML). Macrophages from both mouse strains internalized more parasites from patients with LCL than those with ML (Fig. 3). Additionally, the percentage of ML-infected macrophages decreased after 72 h independent of IFN-γ, whereas the percentage of macrophages infected with parasites from LCL decreased only when macrophages were stimulated with IFN-γ (Fig. 3A and B). Depletion of NO by L-NAME abrogated the IFN-γ independent killing of parasites from patients with ML. A similar effect was seen in macrophages from iNOS KO mice infected with parasites from patients with ML.

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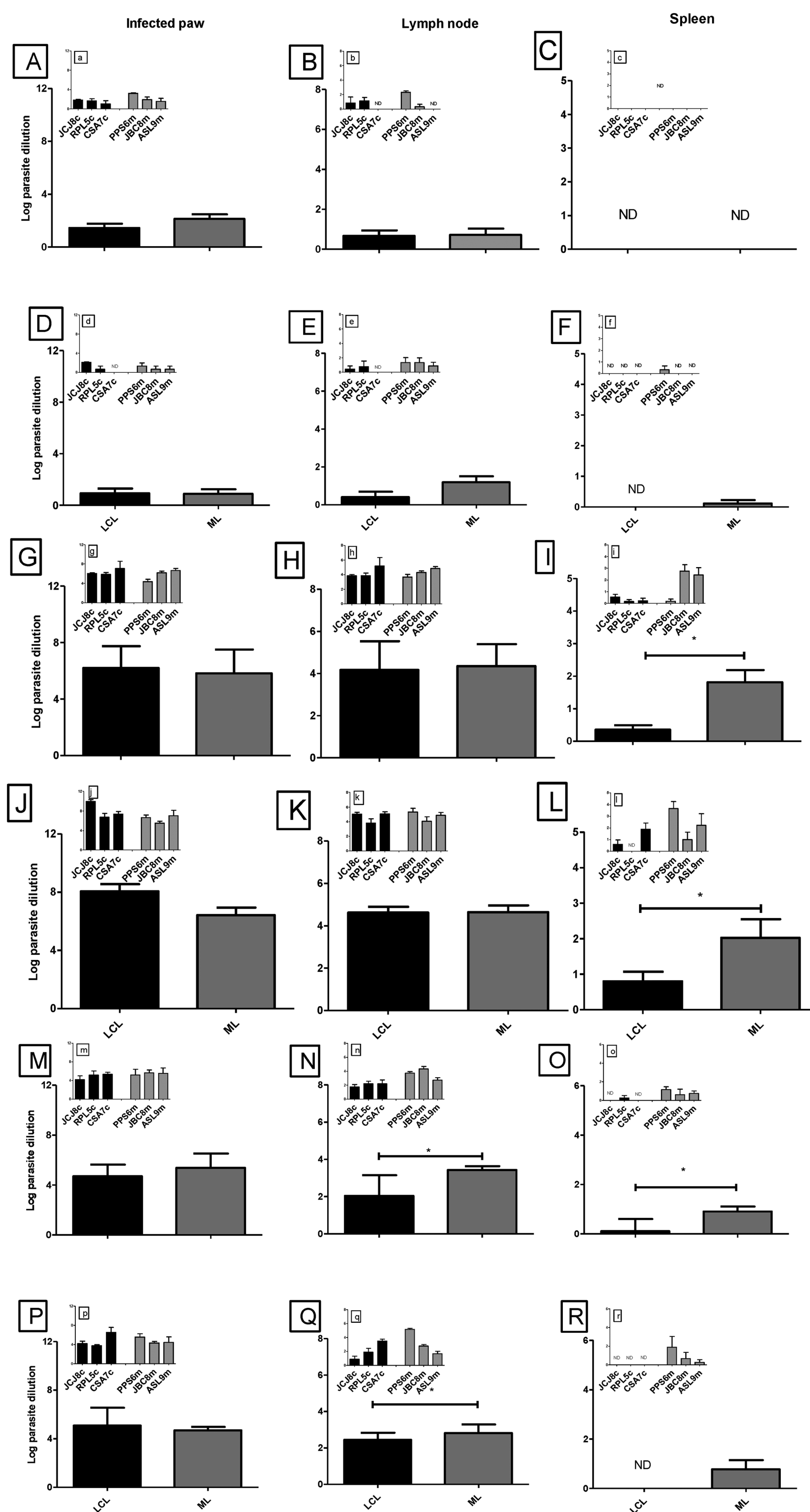

Figure 2. Parasite burden in the paw, draining lymph node and spleen of WT, IFN-γ KO and iNOS KO mice infected with amastigote parasites. WT (**A–F**), IFN-γ KO (**G–L**) or iNOS KO (**M–R**) were infected with 1000 (A, **B**, **C**, G, **H**, **I**, M, **N** and **O**) or 100 000 (**D**, **E**, F, **J**, **K**, L, **P**, **Q**, **R**) amastigotes of *L. (V) braziliensis* isolated from patients with LCL (JCJ8c, RPL5c or CSA7c, black bars) or ML (PPS6m, JBC8m or ASL9m, gray bars). Mice were euthanized as indicated in Figs S1 and S2 (Supporting Information) and the paw (A, D, G, J, M and P), popliteal lymph node (B, E, H, K, N and Q) and spleen (C, F, I, L, O and R) were harvested for assessment of parasite burden by limiting dilution. The data represent the mean ± SD of the parasite burden in each organ from three experiments with three animals per group. Inserts presented the parasite burden for each isolate. Asterisks indicate difference between the parasite burden in each organ by unpaired *t* test ($P < 0.05$). ND: not detected.

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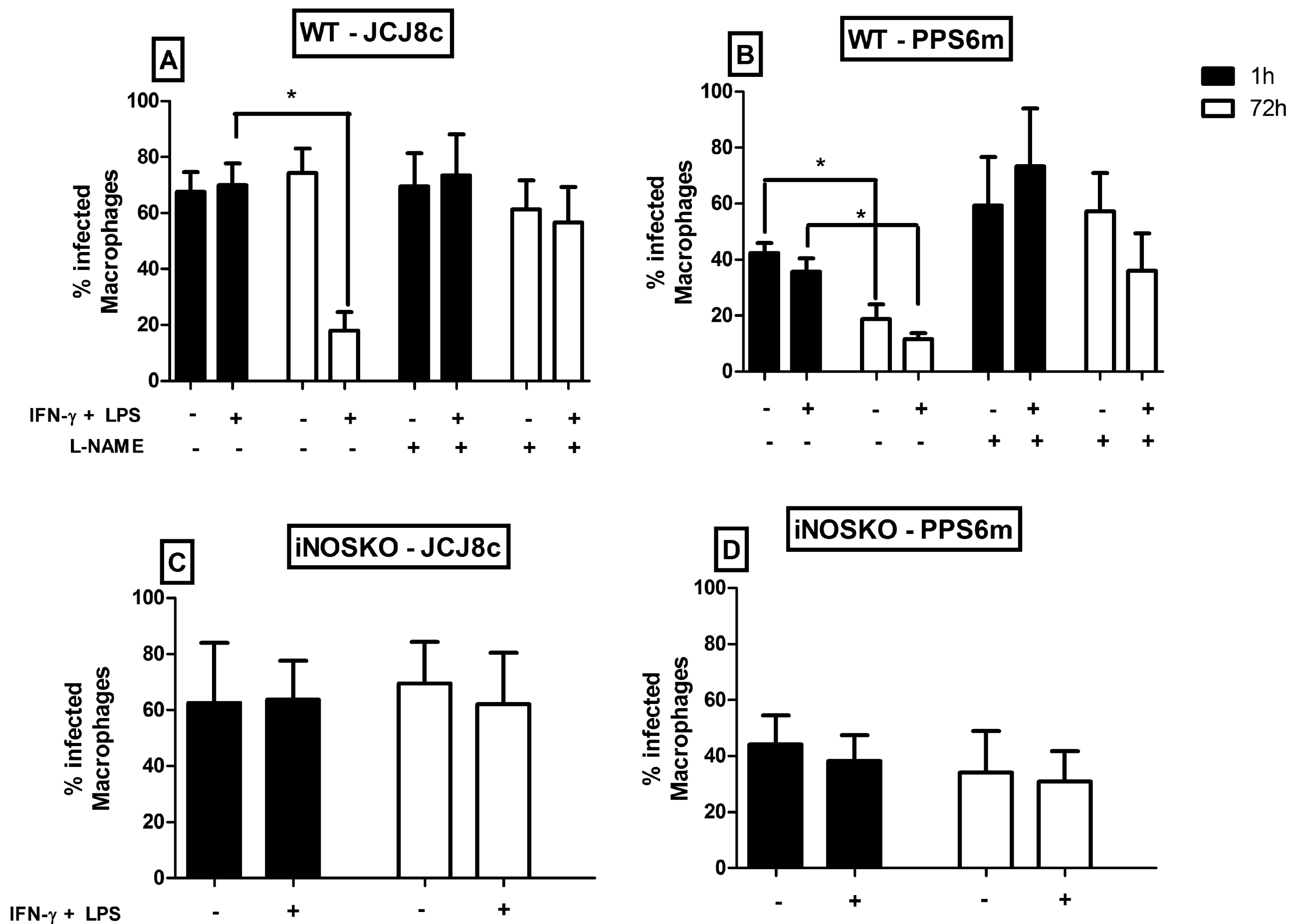

Figure 3. Phagocytic and microbicidal activity of BMDM. BMDM from WT (**A** and **B**) or iNOS KO (**C** and **D**) were infected with *L. (V.) braziliensis* isolated from patients with LCL (JCJ8c) or ML (PPS6m) at the ratio 10:1 (amastigotes: macrophage) in the presence or absence of 10 ng mL^{-1} of IFN-γ, 1 μg mL^{-1} LPS and 2.5 mM of L-NAME. Cells were cultured with amastigotes for 1 h to evaluate phagocytosis (black bars) or 72 h to evaluate microbicidal activity (white bars). The data represent the mean ± SD of the percentage of infected cells from three independent experiments. Asterisks indicate difference by unpaired t test ($P < 0.05$).

Histological characteristics of infected mice euthanized as showed in Figs S1 and 2 (Supporting Information) were investigated and here were presented the data obtained from the paw infected with 1×10^3 amastigotes. The tissue of WT mice infected with parasite from patients with LCL (JCJ8c) maintained its integrity (Fig. 4E, Table S1, Supporting Information) while tissue infected with parasite from patients with ML (PPS6m) presented discreet mononuclear cell infiltration in the dermis (Fig. 4F, Table S1).The IFN-γ KO mice infected with JCJ8c parasites showed accentuated mononuclear cell infiltration, moderated number of infected cells, presence of parasites outside of the cells and mild fibrosis (Fig. 4A, Table S1). This mouse strain infected with PPS6m parasites showed hyperemia, accentuated number of infected macrophages, accentuated mononuclear cell infiltration and mild fibrosis (Fig. 4B, Table S1). Infection of iNOS KO mice with JCJ8c showed moderated hyperemia and mononuclear cell infiltration, discreet number of infected macrophages and discreet neoangiogenesis (Fig. 4C, Table S1). This mouse strain infected with PPS6m parasite presented accentuated number of mononuclear cells infiltrated and infected macrophages, caseous necrosis areas and crust ulceration (Fig. 4D, Table S1).

Because our *in vivo* data suggested that parasites from patients with ML were more sensitive to NO, we investigated the *in vitro* susceptibility of amastigotes to NO. Parasites from LCL and ML were cultured in the presence of 25 or 100 mM of SNP, a NO donor. Amastigotes from patients with ML were more susceptible to NO than parasites from patients with LCL at 25 mM of SNP, whereas no difference was observed at 100 mM (Fig. 5). Additionally, we tested if amastigotes from patients with ML or LCL stimulate NO production in BMDM (Fig. 6) and observed that both parasites were unable to stimulate NO production *in vitro*; however, they inhibited NO production stimulated by IFN-γ + LPS at similar levels.

DISCUSSION

ML appears, most often, after the healing of a primary cutaneous lesion as a metastasis (Marsden 1986). Amastigote is the parasite form present in the vertebrate host when primary lesion is healing, whereas promastigotes are present only in the initial phase of infection. Herein, we used amastigotes initially obtained from patients with LCL or ML to search for differences in the murine infection. After isolation of parasites, we have followed up the patients with cutaneous lesion for more than 6 years and until now, none of them developed ML, suggesting that the parasites from LCL used in this study are related only to cutaneous lesion. However, we cannot exclude the possibility of some parasite from patients with LCL to be able to cause mucosal disease in a patient with susceptible background.

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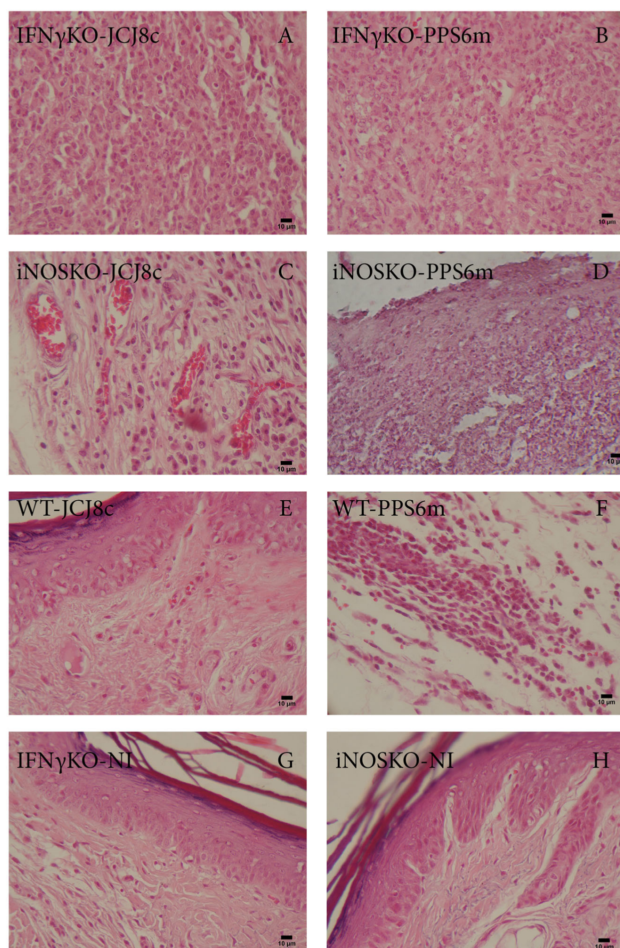

Figure 4. Inflammatory process in the site of *L. braziliensis* infection of IFN-γ KO (**A** and **B**), iNOS KO (**C** and **D**) and WT (**E** and **F**) mice with 1000 amastigotes of *L. (V.) braziliensis* isolated from patients with LCL (JCJ8c—A, C and E) or ML (PPS6m—B, D and F) parasites. Mice were euthanized as indicated in Figs S1 or S2 (Supporting Information) and the paws were collected and processed for histological analysis and HE staining. Uninfected paws of IFN-γ KO (**G**) and iNOS KO (**H**) were shown as control. The horizontal bars represent 10 μm.

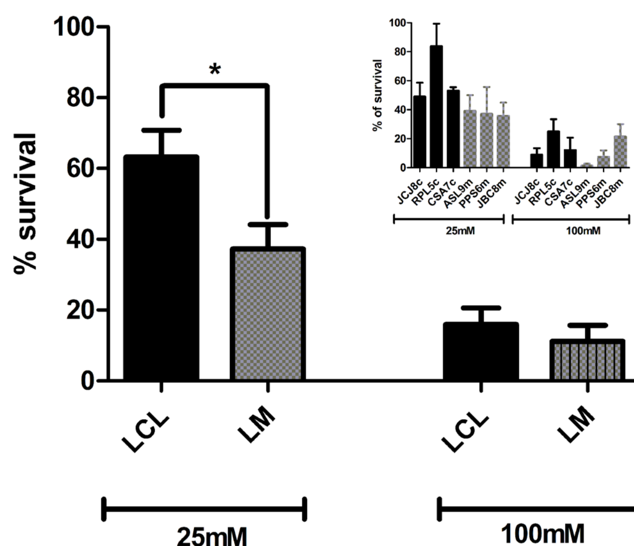

Figure 5. Effects of SNP on the viability of amastigotes parasites. Amastigotes from patients with LCL (JCJ8c, RPL5c or CSA7c) or ML (PPS6m, JBC8m or ASL9m) were cultured for 2 h in the presence of 25 mM or 100 mM of SNP in Grace's medium at 260°C. The viability of parasites was assessed after 20 h by MTT assay. The data represent five independent experiments. Inserts presented the effect of SNP for each isolate. Asterisk indicates difference by unpaired t test ($P < 0.05$).

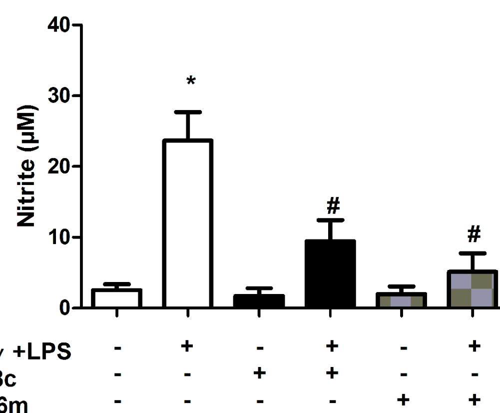

Figure 6. Inhibition of nitric oxide production by amastigotes. BMDM from WT mice were infected or not with *L. (V.) braziliensis* isolated from patients with LCL (JCJ8c) or ML (PPS6m) at the ratio 10:1 (amastigotes: macrophage) in presence or absence of 10 ng mL^{-1} of IFN-γ and 1 μg mL^{-1} LPS. Cells were cultured for 48 h and supernatant was collected for nitrite measurement by using the Griess assay. The data represent the mean $\pm$ SD of the nitrite produced in three independent experiments. Asterisk indicates difference by unpaired *t* test between cells stimulated or not with IFN-γ + LPS and hashes indicate difference between stimulated cells with or without amastigotes ($P < 0.05$).

It was demonstrated here that IFN-γ KO or iNOS KO mice infected with *L. (V.) braziliensis* amastigotes obtained from patients with LCL or ML developed progressive lesions, as shown before for the infection with *L. (V.) braziliensis* promastigote (de Souza-Neto *et al.* 2004; Rocha *et al.* 2007; Oliveira *et al.* 2010). We also observed that the development of lesions in IFN-γ KO mice infected with low dose of parasites from patients with LCL appeared earlier than lesions caused by parasites from patients with ML. In agreement with our findings, it was shown before that promastigotes from patients with LCL induced bigger lesions than parasites from patients with ML in the initial phase of infection of WT C57BL/6 or BALB/c mice (Leite *et al.* 2012; Alves-Ferreira *et al.* 2015). We also observed here that, on the third week of infection, the lesions caused by amastigotes from patients with LCL in WT C57BL/6 mice were bigger than lesions caused by amastigotes from patients with ML, but differences did not reach statistical significance. These data together strongly suggest that lesions caused by parasites from patients with ML develop lesions more slowly than parasites from patients with LCL and the absence of IFN-γ expands the differences. Interestingly, parasites from patients with ML and LCL caused similar lesion in iNOS KO mice at the beginning of infection. Additionally, the lesions in iNOS KO mice caused by parasites from patients with ML appeared earlier than the lesions in IFN-γ KO mice. These data support the major role of NO in controlling murine *L. (V.) braziliensis* infection and suggest that the control of amastigotes from patients with ML at the beginning of murine infection is more dependent on NO than the control of parasites from patients with LCL. NO is the main molecule described as responsible to kill *L. (V.) braziliensis* in mice (Rocha *et al.* 2007) and it is mainly produced after activation of macrophages with IFN-γ. However, NO can also be produced by macrophages stimulated with tumor necrosis factor-α, type I interferons, IL-1 and

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pathogen-associated molecular patterns independent on IFN-γ (Bogdan 2000; Lima-Junior *et al.* 2013).

The better ability of NO in controlling amastigotes from patients with ML than those with LCL was confirmed here by the higher sensitivity *in vitro* of amastigotes from patients with ML to the treatment with NO donor SNP. A higher ability of parasites from patients with ML to induce NO independent of IFN-γ would also justify the small lesion observed in IFN-γ KO mice in the early phase of infection. However, we were unable to detect significant production of NO after *in vitro* stimulus of macrophages with amastigotes from patients with ML or LCL. Additionally, amastigotes from patients with ML or LCL inhibit similarly the NO production in macrophages stimulated with LPS and IFN-γ. Importantly, we observed a killing of parasites from patients with ML, but not from patients with LCL, after infection of macrophages in the absence of IFN-γ and this killing was abolished after inhibition of NO.

The great susceptibility of parasites from patients with ML to NO is in disagreement with some previous studies showing *L. (V.) braziliensis* isolates from patients with ML as more resistant to NO and able to induce less production of this molecule than parasites from patients with LCL (Giudice *et al.* 2007; Campos *et al.* 2008). Additionally, it was observed that most ML isolates hydrolyze more ATP, ADP and AMP, which can decrease NO production (Leite *et al.* 2012). It is important to note that there are differences in the phagocyte interaction with amastigotes and promastigotes what can difficult some comparisons with literature results, mostly with promastigotes. For instance, while promastigotes are taken up by a CR1-mediated phagocytosis involving flagellum, amastigotes are mainly taken up by CR3-mediated endocytosis (Wenzel *et al.* 2012). Amastigotes are not internalized by human neutrophil but are better internalized than promastigotes by human macrophages (Wenzel *et al.* 2012). Amastigotes are more resistant to toxic effects of NO than promastigotes (Genestra *et al.* 2008). We recently showed that *L. braziliensis* amastigotes are able to induce the production of IL-1β, IL-6 and TGF-β, while promastigotes did not (Gomes *et al.* 2014). The amastigotes used here were obtained from infected animals and they probably were opsonized with murine antibodies. Internalization of opsonized particles can favor the activation of regulatory macrophages (Mosser and Edwards 2008) what can alter the initial phase of infection.

A recent paper analyzing promastigotes demonstrated an increased expression of prostaglandin f2-alpha synthase (PGF2S) in parasites from patients with LCL as compared to parasites from patients with ML (Alves-Ferreira *et al.* 2015). High expression of PGF2S was related to an increased *in vitro* infectivity and survival of *L.(V.) braziliensis* and *L. (L.) infantum*, probably by increasing resistance to NO (Dea-Ayuela, Ordonez-Gutierrez and Bolas-Fernandez 2009; Alves-Ferreira *et al.* 2015). These results support our findings suggesting a higher susceptibility to NO and lower infectivity of parasites from ML as compared with those from LCL.

In the late phase of infection, it was suggested that parasites from patients with LCL cause smaller lesions than ML parasites (Leite *et al.* 2012). In our experiments, we observed smaller lesions triggered by parasites from patients with LCL in the late phase after infection of iNOS KO mice. It points out for the action of other microbicidal compound than NO in the late phase of infection with parasites from patients with LCL. The observation that the lesion caused by parasites from LCL reached 2.0 mm on the sixth week in IFN-γ KO and only on the eighth week it reached this size in iNOS KO mice, support that this other microbicidal mechanism is dependent on IFN-γ. IFN-γ is produced by NK cells in the initial stage of murine infection by *Leishmania* parasites, however, the control of the disease is probably more dependent on the IFN-γ produced in the late phase by Th1 lymphocytes (Scharton and Scott 1993; Gazzinelli *et al.* 1998; Liese, Schleicher and Bogdan 2008). Beyond inducing NO production, IFN-γ is able to stimulate ROS production, other important leishmanicidal molecules produced by macrophages (Horta *et al.* 2012). IFN-γ can also induces the expression of idoleamine 2,3-dioxygenase (IDO) (Munn 2006; Lee *et al.* 2013), and this enzyme mediates the degradation of tryptophan, an important amino acid used in the parasite metabolism (Mellor and Hunn 1999). The role of ROS and IDO in infection by parasites of patients with ML and LCL will be evaluated in future studies.

The lesion size is related to the local parasite burden and the inflammatory reaction (Oliveira *et al.* 2012). To evaluate whether the lesion size reflects more the number of parasites at the site of the infection or the inflammatory process, mice were euthanized in different time points when the lesion reached the same size. The data obtained with this procedure showed similar amount of parasites in the paw of all mouse strains when lesion reaches 2.0–3.0 mm. It suggests that the differences observed in the size of the lesion were caused mainly by differences in the parasite burden. Additionally, we detect more parasites in spleen and draining lymph node of mice-infected amastigotes from patients with ML than those with LCL, showing the increased ability of amastigotes from patients with ML to disseminate. It is not clear, at this moment, why amastigotes from patients with ML disseminates more than amastigotes from patients with LCL. However, previous study with metabolomic analyses using promastigotes from patients with ML and LCL showed that these parasites have a differential production of metabolites related to cells chemotaxis (Alves-Ferreira *et al.* 2015), what could justify the difference in the ability of these amastigotes to disseminate.

The analysis of the infected tissue, in the late phase, confirmed that the infection by different isolates causes similar inflammatory response. However, the inflammation was slightly more severe in mice infected with isolates from patients with ML than those with LCL. This last result is consistent with histological characterization of ML in humans, where it observed an intense inflammatory infiltrate and necrotic lesions (de Magalhaes *et al.* 1986; Silveira, Lainson and Corbett 2004).

Our data suggest that amastigotes from patients with ML have decreased the ability to be internalized by macrophages and grow slowly than parasites from patients with LCL; however, they have increased the ability to disseminate and cause more severe infection in the late phase. This data are in agreement with the observation that patients prone to develop ML may have an insufficient immune response in the cutaneous stage, what could be partially responsible for the exacerbated immune response in the late phase (Maretti-Mira *et al.* 2012). Additionally, we observed that the early control of parasites from patients with ML is dependent on NO and less dependent on IFN-γ while parasites from patients with LCL are less sensitive to NO. Because NO produced by human phagocytes is more limited than by murine macrophages, we suggest that the growing of amastigotes from with LCL can be faster in human infection, increasing the possibility to activate a strong immune response at the initial phase of infection to control the parasite. On the other hand, in the initial phase of infection, a small amount of NO produced can limit the growth of parasites that cause ML leading to an insufficient immune response development and failure to control the parasite in the cutaneous stage.

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SUPPLEMENTARY DATA

Supplementary data are available at FEMSPD online.

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AUTHORS' CONTRIBUTIONS

MAPO, CMG, LRA, FRD, conceived the study, LIAP, FDT, PGO, MLD worked in the isolation and maintenance of amastigotes and infected mice; FDT, JCS, CMG and LRA worked in the parasite burden assays; CMG and RSLjr worked in the Histopathological analysis, MAPO, CMG, LRA, FRD analyzed the data and wrote the manuscript. All authors read and approved the final version of the manuscript.

Conflict of interest. None declared.

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