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Research brief

# Hairless mice as an experimental model of infection with *Leishmania* (*Leishmania*) *amazonensis*

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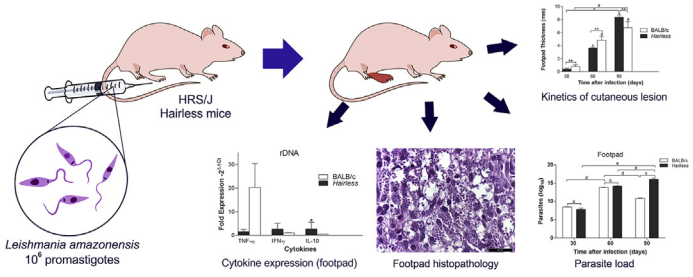
## HIGHLIGHTS

- Hairless mice were susceptible to *Leishmania amazonensis* infection.
- A progressive increase in parasite load and size of lesion was detected over time.
- Expression of IL-10 gene was higher in Hairless than in BALB/c mice.

## GRAPHICAL ABSTRACT

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## ABSTRACT

HRS/J Hairless mice have been investigated as an experimental model in cutaneous leishmaniasis induced by *Leishmania* (*Leishmania*) *amazonensis*. The animals were inoculated with $10^6$ promastigotes into the right hind footpad and the course of infection was followed up for 30, 60 and 90 days. BALB/c mice were infected and used as control. Hairless mice were susceptible to *L.* (*L.*) *amazonensis* infection and a progressive increase in number of parasites and footpad thickness was detected over time. Signals of dissemination and visceralization were confirmed by the presence of parasite in the draining lymph node of lesion and spleen, at different times post infection. IL-10 gene expression evaluated by RT-PCR was significantly higher in Hairless mice at 60 days post infection, corroborating the pattern of susceptibility. These results point this inbred strain as a promising susceptible model for the study of experimental infection induced by *L.* (*L.*) *amazonensis*. This model would allow the use of other infection

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sites that minimize secondary interference and best monitoring the skin lesion, as in the case of *in vivo* assays of potential drugs for LT.



## 1. Introduction

Leishmaniases are a group of non-contagious infectious disease caused by protozoal parasites of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) transmitted to humans through the bite of phlebotomine sand flies (Monzote, 2009; WHO, 2010). Cutaneous leishmaniasis (CL) has a wide spectrum of manifestations, evolving into chronic skin lesions or possible spontaneous healing (Brasil, 2010). These clinical manifestations of CT are associated with specific characteristics of each host, with *Leishmania* species and also with the immune response of the infected individual (Reithinger et al., 2007). *Leishmania* (*Leishmania*) *amazonensis* is one of the etiological agents of American Cutaneous Leishmaniasis (ACL), causing skin lesions associated with Localized Cutaneous Leishmaniasis (LCL) or, in anergic individuals, multiple non-ulcerative nodules (Diffuse Cutaneous Leishmaniasis – DCL) (Silveira et al., 2004; Azeredo-Coutinho et al., 2007).

Murine cutaneous leishmaniasis has emerged as a model to study the development and control of cell-mediated immune response, and susceptibility to *Leishmania* is genetically determined among the various strains of mice (Pearson and Wilson, 1988; Sacks and Noben-Trauth, 2002). Since then, these models are important for both the study and understanding of the pathogenesis and the development of vaccines (Brodskyn et al., 2003; Oliveira and Barral-Neto, 2005; Pereira and Alves, 2008).

CL has been generally studied with experimental models using subcutaneous or intradermal routes, in the footpad, base of the tail or in the ear (Loría-Cervera and Andrade-Narváez, 2014; Moura et al., 2010; Wege et al., 2012). These models have been used in *in vivo* tests of new drugs for the treatment of CL, by oral or intralesional administration, as well as tattooing (Minodier and Parola, 2007; Shio et al., 2014; Tiuman et al., 2012). All these works, however, have been performed using hair-bearing mice.

Mutant Hairless mice present progressive loss of hairs. They have been widely used to study the poor cycle of hairs, and also to test products that reduce skin aging, the damage caused by UVB rays and by treatment with acids, as well as the study of healing of inflammation-associated wounds (Benavides et al., 2009; Massironi et al., 2005; Perez et al., 2012). Hairless mice are a promising model for the study of CL once the disadvantages of the presence of hair would be minimized, such as secondary infections and discontinuity caused by the hair removal. They could provide different sites of infection, optimizing the monitoring of the development of cutaneous lesion and the evaluation of treatment schedules, besides avoiding potential hypersensitivity reactions arising from the epilation.

The aim of this work was the investigation of HRS/J Hairless mice as an experimental model in the CL induced by *L.* (*L.*) *amazonensis*. In the present study, these animals showed a pattern of susceptibility that would be a tool to elucidate important aspects of host–parasite relationship, once this species is associated to an important clinical manifestation in anergic individuals in South America.

## 2. Materials and methods

### 2.1. Animals

Fifty five male inbred HRS/J mice, known as Hairless, were used as experimental model of infection. The animals were obtained from the animal facility of the campus of Ribeirão Preto, University of São Paulo (USP, Brazil), and kept in mating regime at the central animal facility of the Center for Biological and Health Sciences (CCBS) of the Federal University of Mato Grosso do Sul (UFMS, Brazil). Mice aged 4–6 weeks were maintained in individually ventilated cages equipped with mini-isolators, fed a balanced feed (Nuvilab CR-1, Nuvital®) with free access to water. This study received approval from the local Animal Experimentation Ethics Committee (CEUA/UFMS) under protocol number 412/2012.

### 2.2. Parasites

A standard strain of *L.* (*L.*) *amazonensis* (IFLA/BR/1967/PH8) was used for the establishment of infection. Amastigotes were routinely isolated from Golden hamsters (*Mesocricetus auratus*) and maintained as promastigotes in Schneider's Insect Medium (Sigma) supplemented with 20% fetal bovine serum (FBS, Cultilab) and 140 μg/mL gentamicin (Sigma) at 25 °C. On the 7th day of cultivation, promastigotes from up to 4–10 serial passages after isolation were used in the experiments. Silva Jr. et al. (2015) showed that the percentage of metacyclic forms of this strain ranged from 38 to 69% on the 6th day of culture as detected by morphometry, flow cytometry, lectin/antibody selection and complement resistance, using 3rd–6th passage parasites.

### 2.3. Infection and monitoring of cutaneous lesion

Mice of both strains were infected subcutaneously (sc) in the right hind footpad with $1 \times 10^6$ *L. (L.) amazonensis* promastigotes ($1 \times 10^6$/0.1 mL/PBS), and divided into groups of five. The kinetics of the cutaneous lesion was evaluated 30, 60 and 90 days post infection (p.i.). Footpad thickness was measured using a caliper with an accuracy of 0.01 mm (Worker®) and was expressed as the difference between the infected and the mean of five non-infected footpads.

### 2.4. Evaluation of parasite load

Parasite load was evaluated at the inoculation site, popliteal draining lymph node, and spleen at different times of infection in five animals. The organs were removed, weighed and homogenized in 1 mL of Schneider's Insect Medium (Sigma) supplemented with 20% FCS (Sigma) and 140 μg/mL gentamicin (Sigma). The limiting dilution assay was performed in duplicate, as previously described (Titus et al., 1985). The parasite load was calculated using the geometric mean reciprocal of positive titers obtained for the homogenate of each organ divided by the respective weight and the number of parasites per nanogram of tissue was then calculated.

### 2.5. Histopathological study

Five animals from each experimental group were used for the histopathological analysis of the infected footpad, draining lymph node and spleen. Five uninfected animals of each strain were used as controls. The organs were removed and fixed in 10% buffered formalin for subsequent embedment in paraffin. Sections (5 μm) were cut on a microtome (Zeiss Hyrax M25) and stained with Hematoxylin-Eosin (HE). The analysis involved the determination of the nature of the inflammatory infiltrate and the presence of parasite forms. Photomicrographs were taken on an image-capturing microscope (Leica DM5500B).

### 2.6. RNA isolation and real-time PCR

60 days after infection, the animals of both strains were euthanized; infected footpads were removed and stored in buffer solution (350 μL TRIZOL®, Invitrogen, Carlsbad, CA). They were mechanically lysed using MagNALyzer® (Roche Molecular Systems). Nanodrop ND-1000 (Thermocientific®) was used to quantify the total amount of extracted RNA, and 1 μg was used for the c-DNA synthesis. The c-DNA was used for detecting cytokines by Real-Time (RT) PCR. The levels of gene expression (INF-γ, TNF-α and IL-10) were calculated using GAPDH as a housekeeping gene. The reaction began with the activation of the enzyme present in Mastermix (SYBR Green®, Invitrogen) for 10 min at 50 °C and 1 min at 95 °C. The cycling occurred with annealing and extension at the same time for 15 s at 95 °C and 1 min at 60 °C, repeated 40 to 50 times. The analysis was calculated in triplicate using ABI Prism 7500 (Applied Biosystems, Inc., Fullerton, CA, USA). Threshold cycles ($C_t$) were normalized according to GAPDH gene amplification determined by $\Delta Ct = C_t$ (target gene) − $C_t$ (GAPDH gene). The relative expression was calculated using $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \Delta C_t$(target) − $\Delta C_t$, compared to uninfected animals (controls). Primers employed herein are described elsewhere, where the PCR assays were validated (Moura et al., 2010; Carneiro et al., 2012; Santos et al., 2012).

### 2.7. Statistical analysis

Footpad thickness, parasite load and gene expression of cytokines were expressed as the mean ± standard deviation (SD) of five animals per group, respectively, and the data were analyzed using ANOVA followed by Tukey post test when the observation occurred intra-lineage (Hairless × Hairless); and Student's *t*-test when inter-lineage (Hairless × BALB/c). Differences were considered significant at $p < 0.05$ (represented by an asterisk).

## 3. Results and discussion

This study presents the first results of the use of murine strain HRS/J (Hairless) as a model in experimental CL induced by *L.* (*L.*) *amazonensis*. To date, the only study using Hairless mice — albino mice of unmentioned genetic background — in experimental CL was carried out by Packchanian (1979), whose preliminary results showed progressive lesions in animals infected with *Leishmania tropica* at the base of tail. The successful infection of Hairless mice is the first step of a study that aims at the development of other sites of infection apart from the footpad, where the cutaneous lesion could be best induced and accompanied with minimized secondary infections. The use of BALB/c mice as controls has provided a first parameter of susceptibility, allowing the comparison of the same infection site in both species.

The inoculation of promastigotes caused a progressive increase in footpad thickness in both strains studied. When considering Hairless lineage, the increase was significant ($p < 0.001$) between the points of infection (30, 60 and 90 days). So was observed with BALB/c mice, with the same level of significance for 30 and 60 days and 30 and 90 days. Comparing both lineages, the footpads of BALB/c mice were significantly greater on Day 30 ($p = 0.0311$) and Day 60 ($p = 0.0041$). However, on Day 90, Hairless mice footpads were thicker ($p = 0.0116$) than those of BALB/c (Fig. 1).

The kinetics of skin lesion at the infection site was similar to that found by Cupolilo et al. (2003), who infected three susceptible strains with *L.* (*L.*) *amazonensis*. All strains presented progressive increase in lesion until 90 days p.i. In our study, Hairless mice demonstrated greater range in footpad thickness, making the distinction between the periods analyzed more accurate. This range would enable researchers to clinically define the best time to use the experimental model for a certain objective, then making the use of Hairless advantageous compared to BALB/c. For example, Costa Filho et al. (2008) used the analysis of the effects of different treatments on cutaneous lesions as a clinical criterion.

The parasite load correlated with the kinetics of lesion at the site of infection, where a progressive and significant increase was observed in Hairless mice (Fig. 2). Parasite load of BALB/c mice increased from 30 to 60 days p.i., but significantly decreased from 60 to 90 days p.i. (Fig. 2). In the draining popliteal lymph node, parasites were detected in both strains, but no significant difference was seen as to the periods (Fig. 2). Nor was a significant difference seen between the strains in different periods. In the spleen, parasites were detected as early as 30 days p.i., although in a small number in both strains. In Hairless mice, a significant increase was observed between 30 and 90 days, which was not seen in BALB/c mice (Fig. 2). This relevant detection of parasites indicates visceralization of infection in Hairless mice, a largely described condition for *L.* (*L.*) *amazonensis*-infected BALB/c mice, as previously mentioned.

Histopathological study corroborated the clinical characteristics of footpad thickness, and demonstrated the high susceptibility of both strains to *L.* (*L.*) *amazonensis* infection. The animals showed parasitism from the first point of infection, becoming intensified over time. Thirty days after infection, Hairless mice footpads had superficial lesions, with parasites already being observed. Regarding the inflammatory infiltrate a predominantly macrophage response was seen, with some lymphocytes and plasma cells. Insofar as the infection progressed (60 days), new

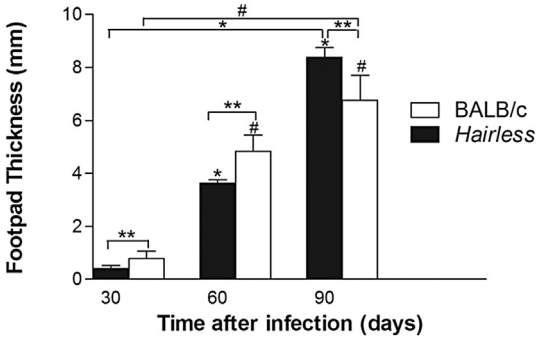


**Fig. 1.** Kinetics of cutaneous lesions induced by *L. amazonensis*. The animals were subcutaneously infected into the right hind footpad with $10^6$ promastigotes. Footpad thickness was measured with a caliper and expressed as the difference between the infected and the mean of five uninfected footpads. Data represent the mean ± SD of five animals per group. *$p < 0.001$ for Hairless 30 × 60 days, 30 × 90 days and 60 × 90 days (ANOVA followed by Tukey post-test). #$p < 0.001$ for BALB/c 30 × 60 days, 30 × 90 days and #$p < 0.05$ for BALB/c 60 × 90 days (ANOVA followed by Tukey's post-test). **$p = 0.0311$ for Hairless × BALB/c 30 days post infection, **$p = 0.0041$ Hairless × BALB/c 60 days post infection and **$p = 0.0116$ for Hairless × BALB/c 90 days post infection (Student's *t*-test).

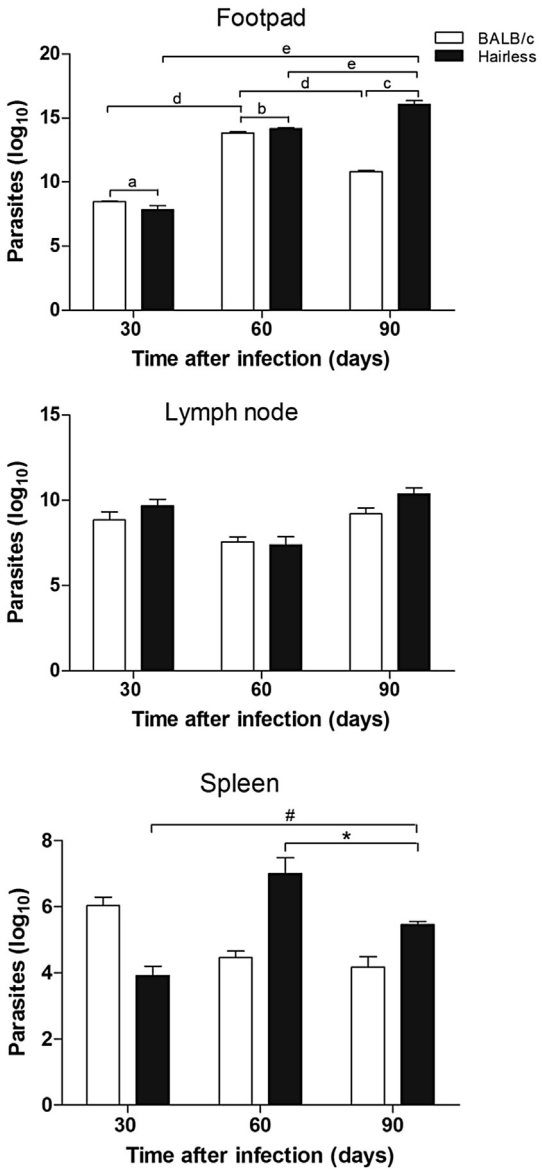


**Fig. 2.** Parasite load in footpads, draining lymph nodes and spleen of Hairless and BALB/c mice. The animals were subcutaneously infected into the right hind footpad with $10^6$ *L. amazonensis* promastigotes. Parasite load was determined 30, 60 and 90 days after infection via a limiting dilution assay. Values represent the mean ± standard deviation of five animals per group. [a]$p = 0.0005$ for Hairless × BALB/c mice − 30 days (Student's *t*-test). [b]$p = 0.0014$ for Hairless × BALB/c mice − 60 days (Student's *t*-test). [c]$p = 0.0304$ for Hairless × BALB/c mice − 90 days (Student's *t*-test). [d]$p < 0.001$ for BALB/c mice, 30 × 60 days; 60 × 90 days (ANOVA followed by Tukey post test). [e]$p < 0.005$ for Hairless 30 × 90 days; 60 × 90 days (ANOVA followed by Tukey post test). [#]$p < 0.01$ for Hairless, 30 × 90 days (ANOVA followed by Tukey post test). [*]$p < 0.05$ for Hairless 60 × 90 days (ANOVA followed by Tukey post test).

tissues were reached and parasitism intensified (Fig. 3A). After 90 days, a disruption of epidermis occurred, giving rise to a typical ulcer. The lesion presented intense diffuse necrosis, macroscopically showing aspect of crust. At this point, a large amount of heavily infected macrophages could be visualized (Fig. 3C). In the lymph nodes, no parasitic forms were observed 60 days p.i. Ninety days after infection, however, a large amount of tissue had been replaced by intense infiltration of parasitized macrophages (Fig. 3E). Similar results were found in BALB/c mice. On Day 30 p.i., the footpads showed discrete lesion, with the presence of infected cells and mild inflammatory infiltrate. After 60 days (Fig. 3B), the lesion had expanded and, 90 days p.i., the entire structure of the footpad was already impaired, showing severe inflammatory infiltrate composed mainly of densely parasitized macrophages (Fig. 3D). Parasitism was discrete in the popliteal lymph node 30 days p.i., with scarce parasitized cells under the capsule, in the cortical region. On Days 60 and 90, parasites were already seen with greater intensity in the latter (Fig. 3F). No alterations were observed in the spleens of infected animals during the experiment. Similar results were described in BALB/c mice by Abreu-Silva et al. (2004), Cupolilo et al. (2003) and Falú et al. (2009).

A significantly higher expression ($p = 0.00181$) of gene IL-10 was observed in the infection site of Hairless mice (Fig. 4). Originally described as an inhibitor of activation of T-helper cells and production of Th1 cytokines (Fiorentino et al., 1989), IL-10 is expressed by various cell types such as macrophages, dendritic cells, B cells and several subsets of cells, such as Th2, regulatory T (Treg) and NK cells (Moore et al., 2001). Thus, this cytokine is classically associated with Th2 profile, which, in leishmaniasis, is related to susceptibility and inability to control the infection (Nylen and Sacks, 2007). The higher expression on IL-10 in Hairless compared to BALB/c mice may be associated to the progressive parasite load in the footpads until 90 days p.i. BALB/c mice showed a decrease in parasite load from 60 to 90 days p.i., but footpad thickness continued to increase, though less than those of Hairless mice. Padigel et al. (2003) showed that BALB/c IL-$10^{-/-}$ mice infected with *L.* (*L.*) *amazonensis* had lower parasite loads in the infection sites (footpad) and draining lymph nodes than wild-type ones, evincing the influence of this cytokine in the control of infection; nonetheless the deficiency in endogenous IL-10 had little effect on lesion development.

The progression of CL is associated with intracellular survival of parasites and deactivation of macrophages (Moura et al., 2005; Fiorentino et al., 1991). In *Leishmania*-susceptible mice the increase of parasitized macrophages are observed with the progressive evolution of lesion. In resistant animals, on the other hand, the increase in number of lymphocytes and the decrease in number of infected macrophages are observed over time, and the inflammatory infiltrate becomes scarce with the resolution of lesion (Andrade et al., 1984; Souza et al., 2000). Homozygous hairless mice (*hr*–*hr*) are known to have about 30% more macrophages (Suzu et al., 2000; The Jackson Laboratories, 2014), which could contribute to the susceptibility pattern of these animals, including the fact that these cells constitute a source of IL-10. Theferore macrophages may play a role in disease exacerbation during the initial stages of *L.* (*L.*) *amazonensis* infection (Pereira and Alves, 2008). Furthermore Hairless mice exhibit a mild immunodeficiency, associated with abnormalities in T-cells (Morrisey et al., 1980; Rezke-Kunz et al., 1979). Zarach et al. (2004) showed that the *Hr* gene encodes a protein highly expressed in skin and brain, which acts as a transcriptional corepressor for some nuclear receptors such as thyroid hormone, retinoic acid, and vitamin D receptors. Mutations in the *Hr* gene could unsettle the transcriptional products of genes related to cell mediated immunity, as showed by Cunliffe et al. (2002). Schaffer et al. (2010) evaluated some cellular and humoral immunological aspects of the SKH1 mouse strain, but the animals were considered as immune competent. Studies should be carried out in order to investigate the role of these proteins on immunological functions of HRS/J Hairless mice, mainly on the gene expression associated to immune response in the skin, where *Leishmania* infection initially takes place. Once the chosen experimental model affects the immunoregulatory events during the infection, Hairless mice may bring some information regarding the immunological profile triggered by *L.* (*L.*) *amazonensis*.

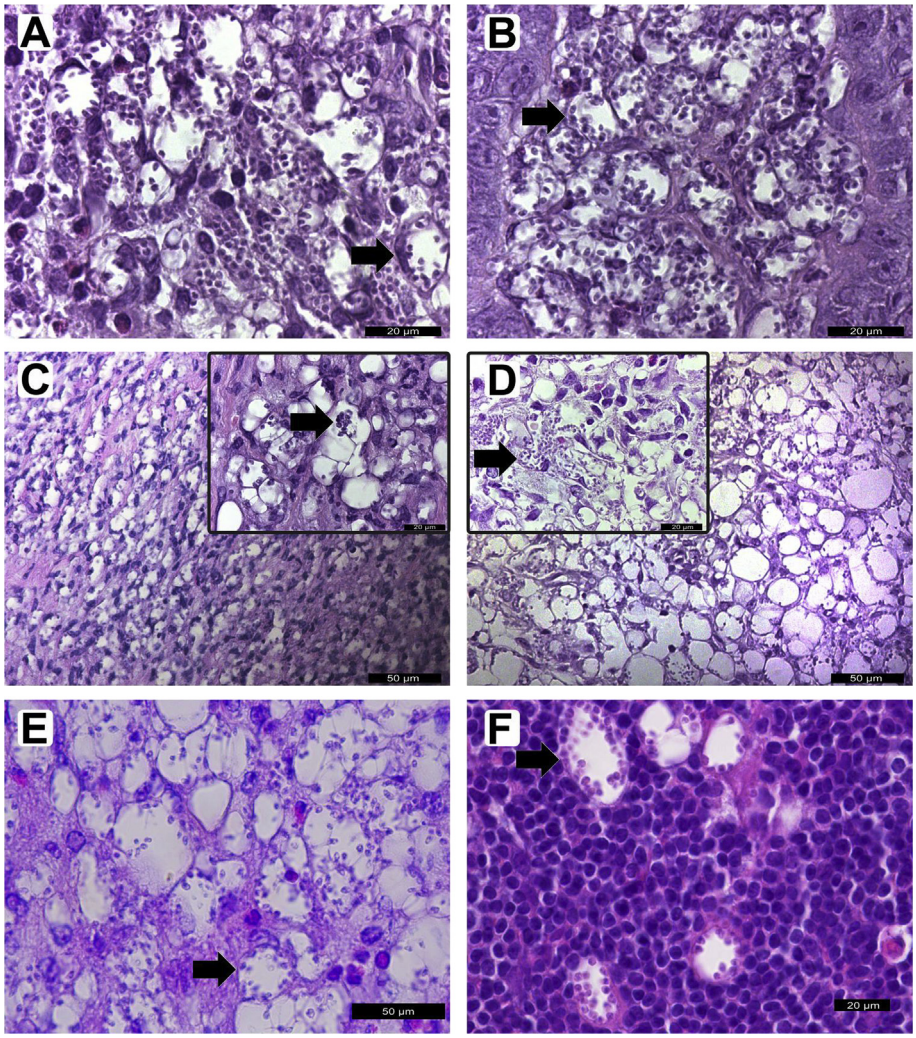


**Fig. 3.** Histopathological analysis of the site of infection and popliteal lymph node in Hairless (A, C, E) and BALB/c (B, D, F) mice. The animals were subcutaneously infected with $10^6$ *L. amazonensis* promastigotes into the right hind footpad. Footpad fragments were obtained 60 (A, B) and 90 (C, D) days post infection. A large number of vacuolated macrophages containing numerous amastigotes was seen in both Hairless and BALB/c footpads (arrows). In popliteal lymph nodes a large number of parasites (arrows) is observed after 90 days in Hairless mice (E). Some parasites are seen at the same point in the lymph nodes of BALB/c mice (F). The figure represents five animals analyzed in each group. HE staining. Magnification: 1000× (A, B, C-window, D-window, E, F); 400× (C, D).

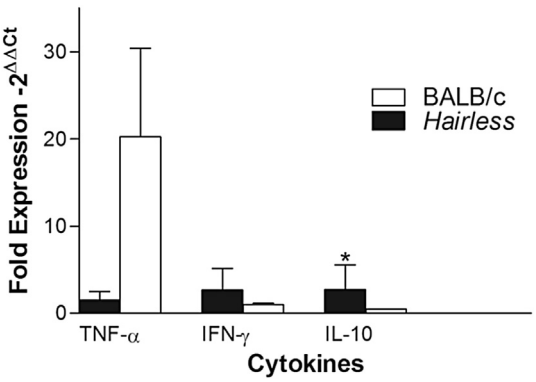


**Fig. 4.** Cytokine expression in the footpads of Hairless and BALB/c mice. The animals were subcutaneously infected into the right hind footpad with $10^6$ *L. amazonensis* promastigotes. Relative quantification of IFN-γ, TNF-α, and IL-10 at the infection site was carried out 60 days after infection. Cytokine cycle threshold ($C_t$) values were normalized to GAPDH expression (housekeeping) as determined by $\Delta C_t = C_{t(\text{cytokine})} - C_{t(\text{GAPDH})}$. Fold change was determined by real-time PCR, using the $-2^{\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = \Delta Ct_{(\text{experimental})} - \Delta Ct_{(\text{control})}$. Data (mean ± SD of three animals per group) represent the fold increase in gene expression of infected over uninfected mice. *$p = 0.00181$ for Hairless × BALB/c mice. Student's *t*-test.

In experimental infection with *L.* (*L.*) *amazonensis*, the predominance of Th1 or Th2 responses cannot be established, due to the different degrees of susceptibility among strains (Abreu-Silva et al., 2004; Diaz et al., 2003; Torrentera et al., 2002; Pereira and Alves, 2008). In inbred mice, several patterns of disease are observed, except in *Leishmania major* infection. The inability to stimulate a proper Th1 response is believed to be responsible for the chronicity of lesions induced by the parasites of *Leishmania mexicana* complex, including *L.* (*L.*) *amazonensis* (McMahon-Pratt and Alexander, 2004). The accurate role of the Th2-type response remains inconclusive, with seemingly contradictory information, especially with respect to the production of some cytokines (Alexander et al., 1999; Brombacher, 2000; Carrera et al., 1996; Guler et al., 1996; Jones et al., 2000; Kropf et al., 1997).

The study of immune response in experimental leishmaniasis is important to the understanding of the mechanisms involved in resistance or susceptibility to different *Leishmania* species. It provides data to clarify the factors responsible for the different clinical forms of the disease and the wide spectrum of possible immune responses, which could contribute to the development of more effective vaccines and treatments against the disease.

## 4. Conclusions

The present study demonstrated the susceptibility of Hairless mice to *L.* (*L.*) *amazonensis* infection, with progressive lesions and chronic persistence of the parasite in the site of infection, besides the susceptibility pattern of expressed cytokines. Signs of proliferation and visceralization were confirmed by the presence of parasites in the spleen and the draining lymph nodes of lesion. Studies are being carried out to establish the cutaneous lesion in other sites of infection in these animals.

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