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Infection by *Leishmania amazonensis* in mice: A potential model for chronic hypoxia

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ABSTRACT

Hypoxia is a common feature of injured and infected tissues. Hypoxia inducible factors 1α and 2α (HIF-1α, HIF-2α) are heterodimeric transcription factors mediating the cellular responses to hypoxia and also the vascular endothelial growth factor (VEGF). VEGF is a cytokine which can be induced by hypoxia, whose pathogenic mechanisms are still unclear and which is the subject of debate. Murine cutaneous lesions during *Leishmania amazonensis* parasite infection are chronic, although they are small and self-controlled in C57BL/6 mice and severe in BALB/c mice. In the present study we examined the presence of hypoxia, HIF-1α, HIF-2α and VEGF during the course of infection in both mouse strains. Hypoxia was detected in lesions from BALB/c mice by pimonidazole marking, which occurred earlier than in lesions from C57Bl/6 mice. The lesions in the BALB/c mice showed HIF-1α and HIF-2α expression in the cytoplasm of macrophages and failed to promote any VEGF expression, while lesions in the C57BL/6 mice showed HIF-2α nuclear accumulation and subsequent VEGF expression. In conclusion, the animal models of leishmaniasis demonstrated a diversity of patterns of expression, cell localization and activity of the main transducers of hypoxia and may be useful models for studying the pathogenic mechanisms of HIF-1α and HIF-2α during chronic hypoxic diseases.

Introduction

Hypoxia (low oxygen tension) can occur during various pathological conditions. Cellular responses to this condition are mainly regulated by the activation of heterodimeric transcription factors, called hypoxia inducible factors (HIFs), which are critical regulators of cellular responses for a variety of stressful conditions. HIFs consist of a constitutively expressed β-subunit and an oxygen-regulated α-subunit (Kaelin, 2005). Under non-stressful conditions, the HIF α-subunit is subjected to enzymatic proline hydroxylation, which targets it for proteasome degradation *via* the von Hippel-Lindau ubiquitin E3 ligase complex (Schoefield and Ratcliffe, 2004). Under stressful conditions the ubiquitination of HIF is inhibited and the α-subunit accumulates as a consequence of prolyl hydroxylase inhibition (Maxwell et al., 1999). The stabilized HIF α-subunit dimerizes with the β-subunit, activating transcription of target genes that regulate angiogenesis and other factors important for responding to stressful conditions such as VEGF and glycolytic enzymes (Fukuda et al., 2003). There are several distinct α-subunits, but the presence of HIF-1α and HIF-2α is more frequently elevated in cancer cells and mainly associated with invasiveness, poor prognosis and angiogenesis (Favier et al., 2007; Imtiyaz et al., 2010).

Evidence suggests that HIF-1α has both pro- and anti-proliferative properties, whereas HIF-2α lacks anti-proliferative properties and is more strongly implicated in tumorigenesis (Gordan and Simon, 2007). Experimental studies, however, have led to discrepancies in results regarding their roles under stressful conditions, indicating varying functions according to the cell type (Kondo et al., 2002; Chen et al., 2003; Favier et al., 2007). Thus, the role of HIF-1α and HIF-2α in a variety of pathological contexts is complex and not fully described (Imtiyaz et al., 2010). To date, few studies have concomitantly evaluated the expression of HIF-1α and HIF-2α (Mowat et al., 2010; Imtiyaz et al., 2010; Yang et al., 2011; Barshishat-Kupper et al., 2011).

A potential experimental model for studying hypoxia and the differential functions of HIFs may include macrophages. These phagocytes are versatile hematopoietic cells that mediate a wide array of immune functions, *e.g.*, initiating inflammatory responses, executing phagocytosis and parasitical killing or association with tumor angiogenesis (Stout and Suttles, 2004; Mosser and Edwards, 2008). When exposed to hypoxia, macrophages accumulate both HIF-1α and HIF-2α. An over-expression of HIF-2α in tumor-associated macrophages is specifically correlated with high-grade human tumors and poor prognosis (Gordan and Simon, 2007).

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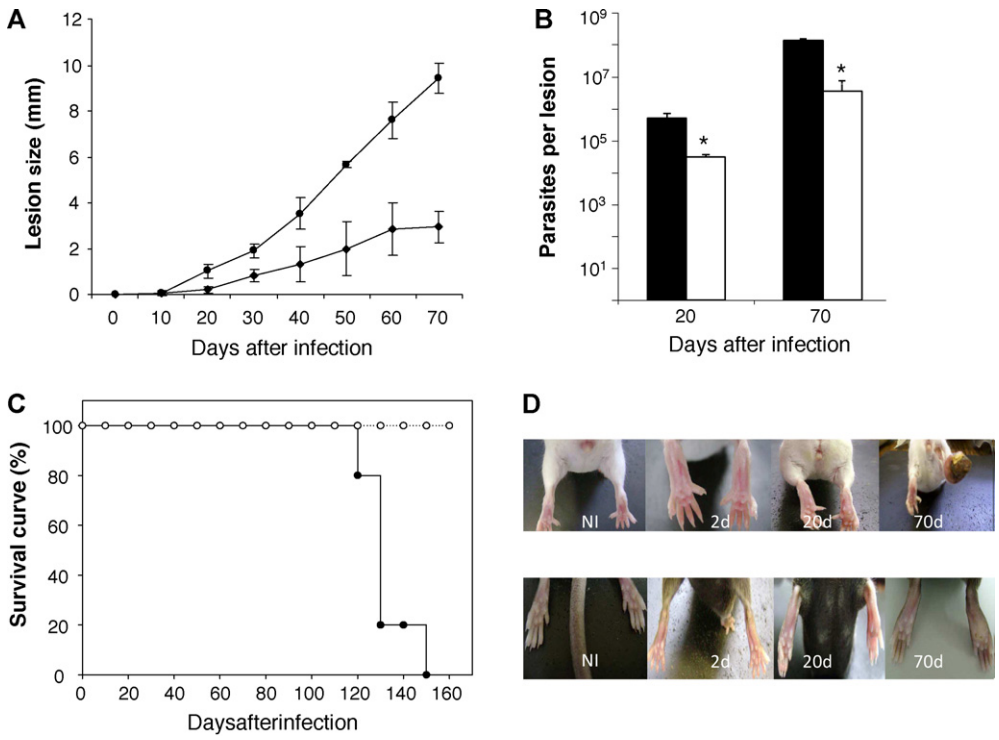

Fig. 1. Analysis of experimental *L. amazonensis* infection. (A) The evolution of infection in mice. Lesion size is expressed as the difference in size between the infected and contralateral non-infected footpad. BALB/c (●) and C57Bl/6 (⧫) mice. $n = 10$. (B) Parasite burden of lesions. At the times indicated three mice per group were sacrificed, infected footpads collected and parasite numbers determined as described in "Materials and methods section". BALB/c (■) and C57Bl/6 (□) mice. (C) Survival curves for mice infected with *L. amazonensis.* BALB/c (●) and C57Bl/6 (○) mice survival percentage. $n = 10$. (D) Evolution of cutaneous infection in mice. NI: non infected mouse. The significance of the difference between experimental conditions is indicated: $^*P < 0.05$ in B.

Leishmaniasis is an endemic parasitosis caused by several species of the genus *Leishmania*, an obligate intramacrophagic parasite (Nylén and Gautam, 2010). In addition, murine experimental leishmaniasis has become a useful model for dermal inflammation (Baumer et al., 2010) and for the study of certain aspects of chronic diseases such as myopathies (Paciello et al., 2010). The cutaneous lesions of mice during *Leishmania amazonensis* infection are chronic although they can be self-controlled in C57BL/6 mice and severe in BALB/c mice (Giorgio et al., 1998). We previously observed HIF-1α expression in lesions from BALB/c mice (Arrais-Silva et al., 2005). In addition, using an *in vitro* system, we demonstrated HIF-1α and HIF-2α expression in infected macrophages (Degrossoli et al., 2007, 2011). The aim of the present study was to examine the presence of hypoxia in both mouse strains during the course of chronic *L. amazonensis* infection and to evaluate the expression patterns of HIF-1α, HIF-2α and VEGF within lesions.

Materials and methods

Parasite and infection

The experimental protocols were approved by the Institute of Biology/Universidade Estadual de Campinas Ethical Committee for Animal Research. *L. amazonensis* (MHOM/BR/73/M2269) was maintained by regular passage in BALB/c mice as described previously (Barbieri et al., 1993). Female BALB/c and C57BL/6 mice (6 weeks old) were obtained from the Centro de Bioterismo-UNICAMP, Campinas, SP, Brazil, and 10^5 amastigotes were injected subcutaneously in the right hind footpad (Arrais-Silva et al., 2005).

Evaluation of infection

The course of the infection was monitored by measuring the increase in footpad thickness, compared with the contralateral uninfected footpad, with a dial caliper (Giorgio et al., 1998). In order to estimate parasite burden in the lesions, mice were sacrificed at designated periods, the entire infected footpads were removed and weighed and amastigotes were recovered from the lesions and counted (Arrais-Silva et al., 2005). The survival rate of the animals was monitored throughout the experimental period. Three independent experiments involving 10 mice each were performed in order to evaluate infection and allow the immunohistochemical study.

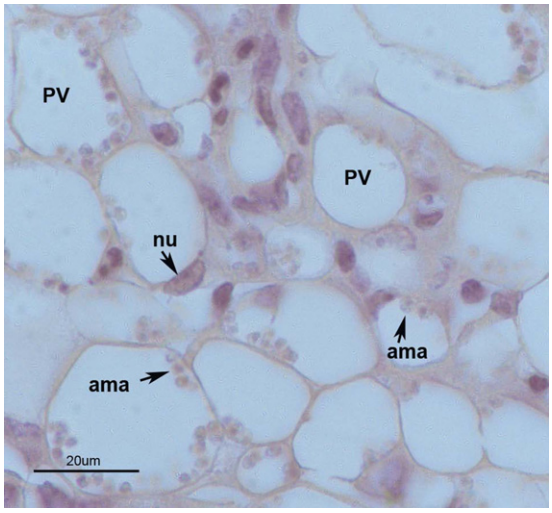

Fig. 2. Immunostaining for macrophages. Representative Mac-3 staining of infected macrophages in footpad lesion of BALB/c mouse infected with *L. amazonensis*. Amastigotes (ama), nucleus (nu), vacuole parasitophorous (PV).

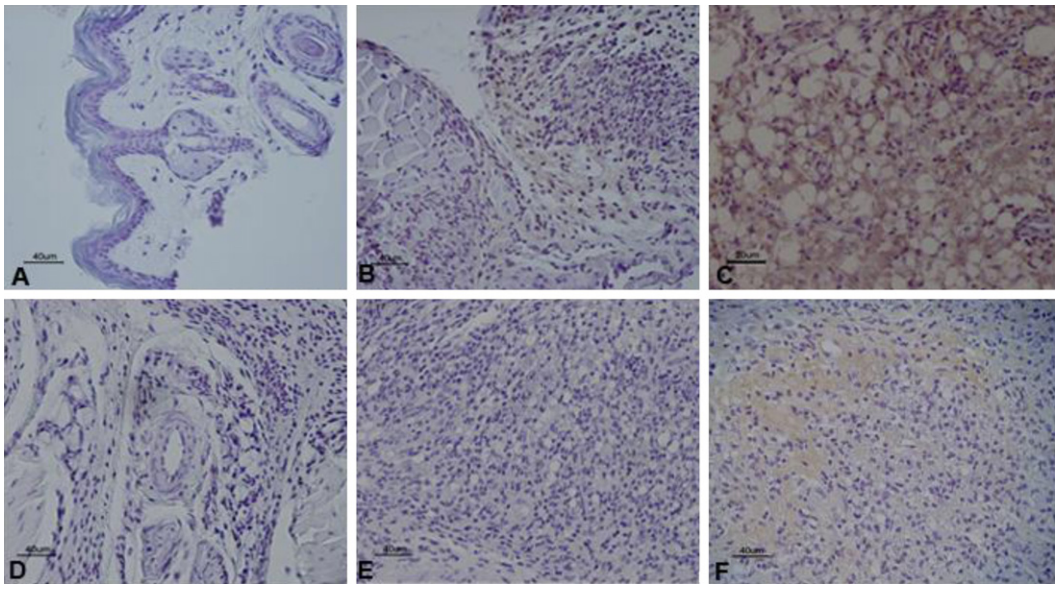

Fig. 3. Evaluation of hypoxia by immunohistochemistry in footpad lesions of mice infected with *L. amazonensis*. Mouse lesion tissue was stained with anti-pimonidazole complex antibody as described in "Materials and methods section". BALB/c mouse lesion at 2 days p.i. (A), 20 days p.i. (B) and 70 days p.i. (C). C57BL/6 mouse lesion at 2 days p.i. (D), 20 days p.i. (E) and 70 days p.i. (F).

Tissue hypoxia analyses

The mice were administered intraperitoneally with pimonidazole hydrochloride (Hypoxyprobe-1, Chemicon Int., Temecula, CA, USA) dissolved in sterile, filtered physiological saline at 60 mg/kg body weight. After 90 min, the footpad lesion was perfusion fixed with 4% paraformaldehyde and embedded in paraffin, followed by immunohistological detection of pimonidazole protein adducts, in accordance with the manufacturer's recommendations and as described previously (Araújo et al., 2010). Briefly, serial 5 μm thick paraffin sections of lesions were treated as follows: deparaffinization, rehydration, endogenous quenching and citrate buffer microwave antigen retrieval. To detect pimonidazole, the sections were incubated with a mouse anti-pimonidazole antibody (Chemicon, Temecula, CA, USA), developed with a secondary polyclonal anti-mouse antibody conjugated with peroxidase (Sigma–Aldrich, St. Louis, MO, USA) and visualized with a peroxidase substrate solution containing 3,3′-diaminobenzidine (DAB) and hydrogen peroxide. Tissue sections were counterstained with hematoxylin, dehydrated in serial alcohol solutions and mounted in cytoseal-60 mounting medium (Sigma–Aldrich). The images were recorded using a digital imaging system, a Nikon light microscope (Eclipse E800, Nikon, Tokyo, Japan), a Cool Snap-Pro Color camera (Media Cybernetics, Bethesda, MD, USA) and the Image-Pro plus capture software (Media Cybernetics).

HIF-1α, HIF-2α immunofluorescence

The footpad tissues from BALB/c and C57BL/6 mice were fixed for 24 h with paraformaldehyde and processed into paraffin blocks. Tissue sections (5 μm) were deparaffinized, dehydrated and the antigen retrieval was performed by using citrate buffer 10 mM (pH 6.0) in a microwave oven. Non-specific binding sites were blocked with 1% bovine serum albumin (Amresco, Dallas, TX, USA) for 30 min. Tissue sections were then incubated with primary antibodies: rabbit polyclonal anti-HIF-1α antibody (H-206) (Santa Cruz) or rabbit polyclonal anti HIF-2α antibody (a gift from Dr. Darren Richard), overnight at 4 °C in a humidified room; followed by an FITC-conjugated goat anti-rabbit secondary antibody (Sigma–Aldrich) for 1 h at room temperature. The tissues were mounted with 4′,6-diamidino-2-phenylindole (DAPI)-containing DABCO mounting media (Sigma–Aldrich). The cells were visualized under a Nikon Eclipse 50i fluorescence microscope (Nikon). All images were captured and analyzed with a digital camera (Nikon DXM1200-F) and imaging software (ACT-1, Nikon).

Immunohistochemistry

Tissue sections were deparaffinized and dehydrated, treated with 5% hydrogen peroxide for 30 min, and washed with PBS. Antigen retrieval was performed by citrate buffer 10 mM (pH 6.0) in a microwave oven. Non-specific binding sites were blocked with 1% BSA (Amresco, Solon, OH, USA) for 30 min. Tissue sections were then incubated with primary antibody rabbit polyclonal anti-VEGF antibody (Chemicon) and with a secondary goat polyclonal anti-rabbit antibody conjugated with peroxidase (Sigma–Aldrich). A rat anti-mouse Mac-3 (BD Biosciences Pharmingen, San Diego, CA, USA) and a secondary goat anti-rat conjugated with peroxidase (Sigma–Aldrich) were used to identify macrophages. Tissue sections were visualized with a peroxidase substrate solution containing 3,3′-diaminobenzidine (DAB) and hydrogen peroxide (Araújo et al., 2010) and counterstained with hematoxylin, dehydrated in graded alcohol solutions and mounted in cytoseal-60 mounting medium (Sigma). The images were recorded as described above.

Statistical evaluation

All experiments were repeated at least three times. The results are expressed as the mean ± SD. Data obtained under different conditions were analyzed statistically by the Student *t*-test, with a level of significance set at $P < 0.05$ for *L. amazonensis* infection assays.

Results

L. amazonensis infection in mice

Mice were inoculated with *L. amazonensis* and lesion progression, tissue parasite burden and survival period were monitored. The lesions from the BALB/c mice increased progressively faster during the course of the infection and most of the animals had

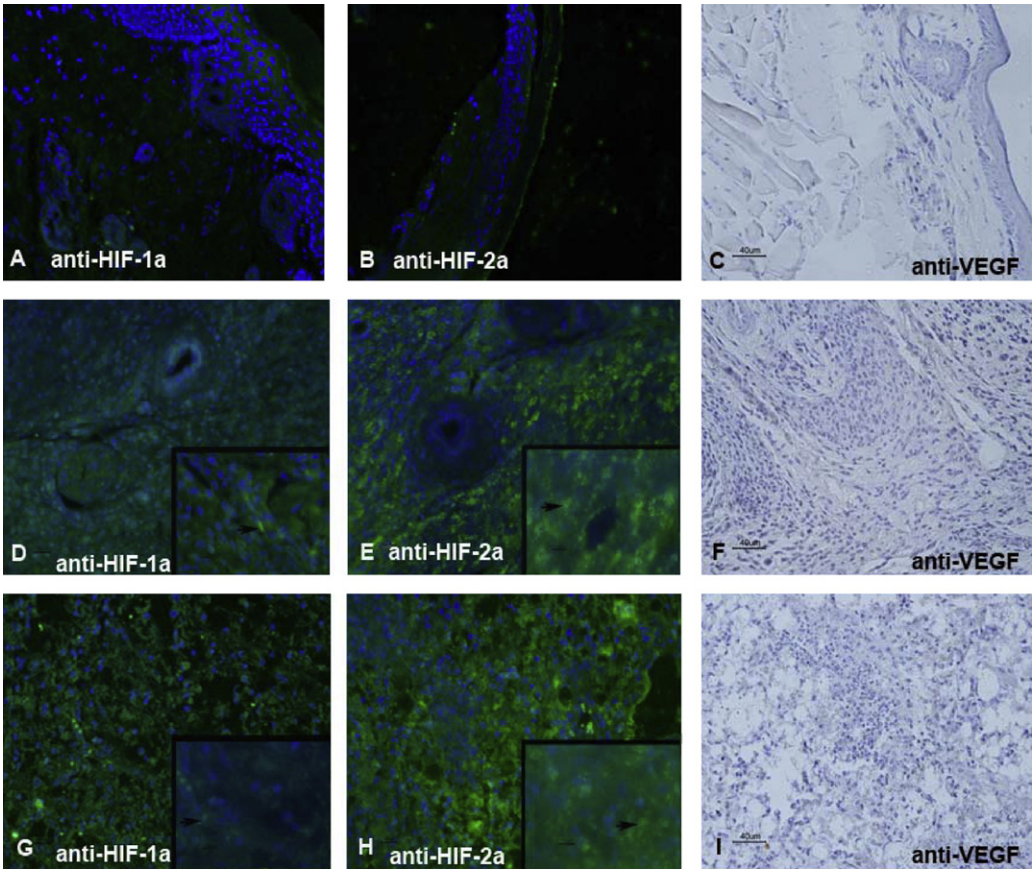

Fig. 4. Evaluation of HIF-1α and HIF-2α by immunofluorescence, and VEGF by immunohistochemistry in footpad lesions of BALB/c mice infected with *L. amazonensis*. Lesion tissue at 2 days p.i. stained with anti-HIF-1α polyclonal antibody (A), anti-HIF-2α polyclonal antibody (B), and anti-VEGF polyclonal antibody (C). Lesion tissue at 20 days p.i. stained anti-HIF-1α polyclonal antibody (D), anti-HIF-2α polyclonal antibody (E), and anti-VEGF polyclonal antibody (F). Lesion tissue at 70 days p.i. stained anti-HIF-1α polyclonal antibody (G), anti-HIF-2α polyclonal antibody (H), and anti-VEGF polyclonal antibody (I). The blue color indicates DAPI staining in the nuclei of macrophages. The green color indicates HIF-1α or HIF-2α in the cytoplasm of macrophages (arrowheads). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

infected footpad skin ulcer sizes ranging from 8.5 to 10 mm at 70 days post-infection (p.i.) (Fig. 1A and D). The mice showed lesions with necrotic areas and bacterial contamination following this period. In contrast, lesions from the C57BL/6 mice progressed slowly and did not display any ulceration (Fig. 1A and D). Parasites were not detectable in lesions at 2 days pi. Although the parasitic burden in both mouse strains increased progressively, the number of parasites found in the lesions from the BALB/c mice was approximately 100 and 10 times-higher than the number of parasites found in the lesions from the C57BL/6 mice, at 20 and 70 days p.i. respectively (Fig. 1B). The BALB/c mice presented metastatic lesions on their ears and/or face, and died after 120 days p.i. while the C57BL/6 mice survived during the entire experimental period (Fig. 1C). These results confirmed previous observations (Giorgio et al., 1998) that BALB/c mice are susceptible and C57BL/6 mice are relatively resistant to *L. amazonensis* infection.

Hypoxia detection in lesions of mice infected with *L. amazonensis*

Hypoxia was evaluated using pimonidazole as a surrogate for hypoxia. Pimonidazole was administered to the mice and 90 min later they were sacrificed and pimonidazole adducts with thiol containing protein were detected by a peroxidase-antibody (Via et al., 2008). Footpad lesions at 2 days p.i. had the epidermis and glandular structures conserved in both mice. No evidence of pimonidazole immunostaining was observed in lesion tissue from either mouse strain (Fig. 2, BALB/c upper row, A–C and C56BL/6, lower row, D–F) at this phase. At 20 days p.i. a cellular population infiltrating the tissue, parasitized macrophages and pimonidazole immunostaining were observed in the lesions of BALB/c mice. Later in the infection (70 days p.i.) lesions showed many pimonidazole positive vacuolated and parasitized macrophages (Fig. 2C). In contrast, lesions from the C57BL/6 mice at 20 days p.i. were characterized by few parasitized macrophages and no pimonidazole immunostaining, however inflammatory cells, parasitized macrophages and pimonidazole immunostaining were observed at 70 days p.i. (Fig. 2F). It should be noted that no significant pimonidazole immunostaining of footpad tissue was observed in uninfected mice (data not shown). Immunohistochemical studies of lesions consistently showed staining of infected macrophages for the macrophage surface antigens Mac-3 (Fig. 3), F4/80 and Mac-1 (McElrath et al., 1987). Macrophages are found to be the predominant infiltrating cell type in the lesions with inflammatory cells polymorphonuclear leukocytes and lymphocytes in smaller numbers during BALB/c mice infection and at 20 days p.i. in C57BL/6; later it consists predominantly of the inflammatory cells (Table 1). These results indicate that *L. amazonensis* infection induces lesions containing hypoxic areas and macrophage infiltration.

HIF-1α, HIF-2α and VEGF expression in lesions of mice infected with *L. amazonensis*

We then addressed whether *L. amazonensis* infection affects HIF-1α and HIF-2α stabilization in mice lesions. At 2 days p.i. there was

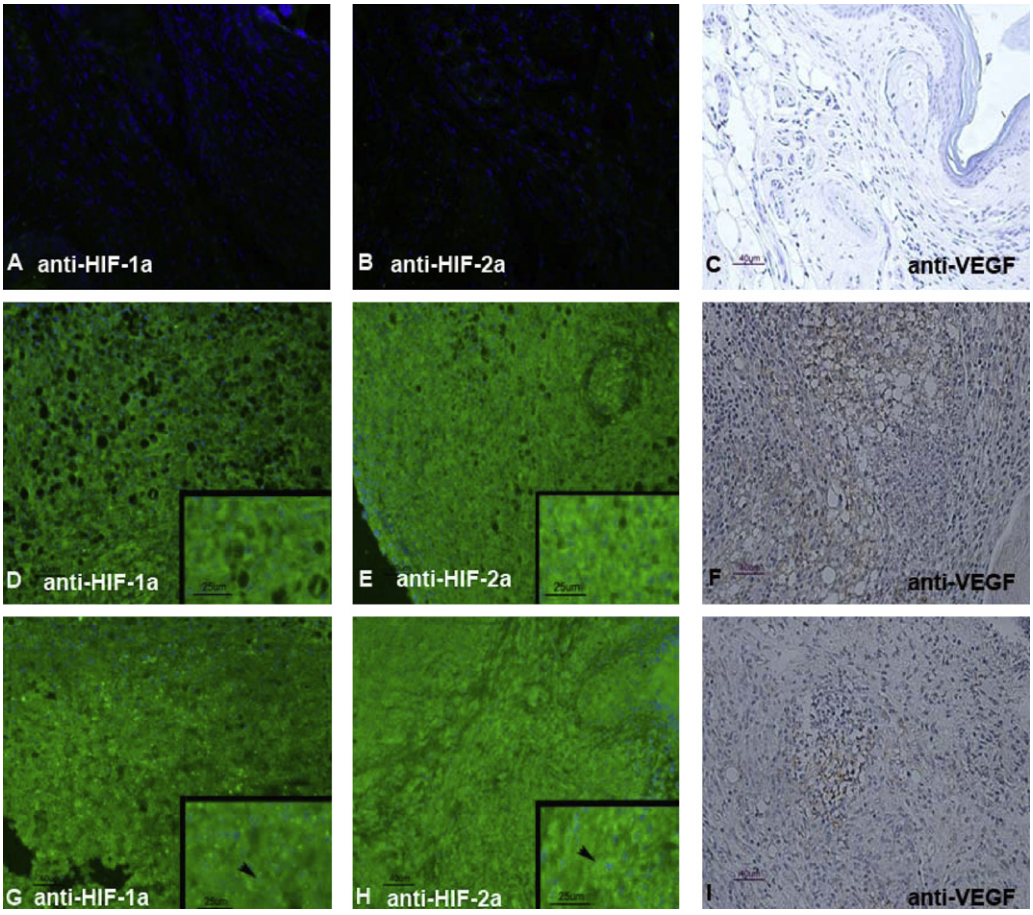

Fig. 5. Determination of HIF-1α and HIF-2α by immunofluorescence, and VEGF by immunohistochemistry in footpad lesions of C57Bl/6 infected with *L. amazonensis*. Lesion tissue at 2 days p.i. stained with anti-HIF-1α polyclonal antibody (A), anti-HIF-2α polyclonal antibody (B), and anti-VEGF polyclonal antibody (C). Lesion tissue at 20 days p.i. stained anti-HIF-1α polyclonal antibody (D), anti-HIF-2α polyclonal antibody (E), and anti-VEGF polyclonal antibody (F). Lesion tissue at 70 days p.i. stained anti-HIF-1α polyclonal antibody (G), anti-HIF-2α polyclonal antibody (H), and anti-VEGF polyclonal antibody (I). The yellow color indicates DAPI and HIF-1α or HIF-2α staining in the nuclei of macrophages (arrowheads). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

no evidence of HIF-1α, HIF-2α or VEGF expression (Fig. 4A–C) in the lesions from the BALB/c mice. The chronic stabilization of infection dramatically increased HIF-1α and HIF-2α expression (Fig. 4 D and E) in the lesions at 20 days p.i. without any evidence of VEGF expression (Fig. 4F). The same staining pattern persisted during the experimental period, *i.e.* HIF-1α and HIF-2α expression without VEGF expression up to 70 days p.i. (Fig. 4G–I) in the lesions of the BALB/c mice and localized predominantly in the cytoplasm of infiltrated cells and parasitized macrophages. In contrast, distinct staining for HIFs was noticed in the lesions from the relatively resistant C57BL/6 mice. As shown in Fig. 5, *L. amazonensis* infection did not induce cellular HIF-1α and HIF-2α stabilization or VEGF expression in the lesions at 2 days p.i. (Fig. 5A–C). On the other hand, during the chronic period the lesions showed HIF-1α, HIF-2α and VEGF expression at 20 days p.i. (Fig. 5D–F) and 70 days p.i. (Fig. 5G–I). Through merged images, the localization of HIF-1α in the cytoplasm and nucleus (Fig. 5D and G) and predominant localization of HIF-2α in the nuclei (Fig. 5E and H) became evident. We also observed that in nuclei, HIF-1α and HIF-2α accumulation persisted at least until 70 days p.i. in infected macrophages.

Table 1
Cell infiltration in lesions of mice infected with *L. amazonensis*.[a]

Mouse strain	Days p.i.	Macrophages[b]	Other cells[c]
Balb/c	20	58	42
Balb/c	70	91	9
C57Bl/6	20	83	17
C57Bl/6	70	33	67

[a] The lesions were stained with hematoxylin and cells were counted in 10 random fields.
[b] Parasitized and non-parasitized macrophages.
[c] Inflammatory cells: polymorphonuclear cells and lymphocytes.

Discussion

The discovery of disease models for studying the role of hypoxia and different subunits of HIF during pathological processes is important to allow the identification of molecular targets in the treatment of cancer and chronic infectious diseases. Previous studies made in various groups demonstrated HIF-1α expression in murine models of infectious diseases such as schistosomiasis, paracoccidiomycosis and respiratory syncytial virus infection (Haeberle et al., 2008; Ferreira et al., 2009; Araújo et al., 2010). However, the *in vivo* models represented the susceptible phenotypes to these infections. The analyses of hypoxia and hypoxia related parameters of susceptible and resistant inbred mouse strains to an experimental infection may allow better understanding of the chronic pathological mechanisms. The experimental infection of mice by *Leishmania* constitutes one of the most studied models of parasitic disease (Nylén and Gautam, 2010). A comparison of clinical and immunological parameters recorded in infected mice with those

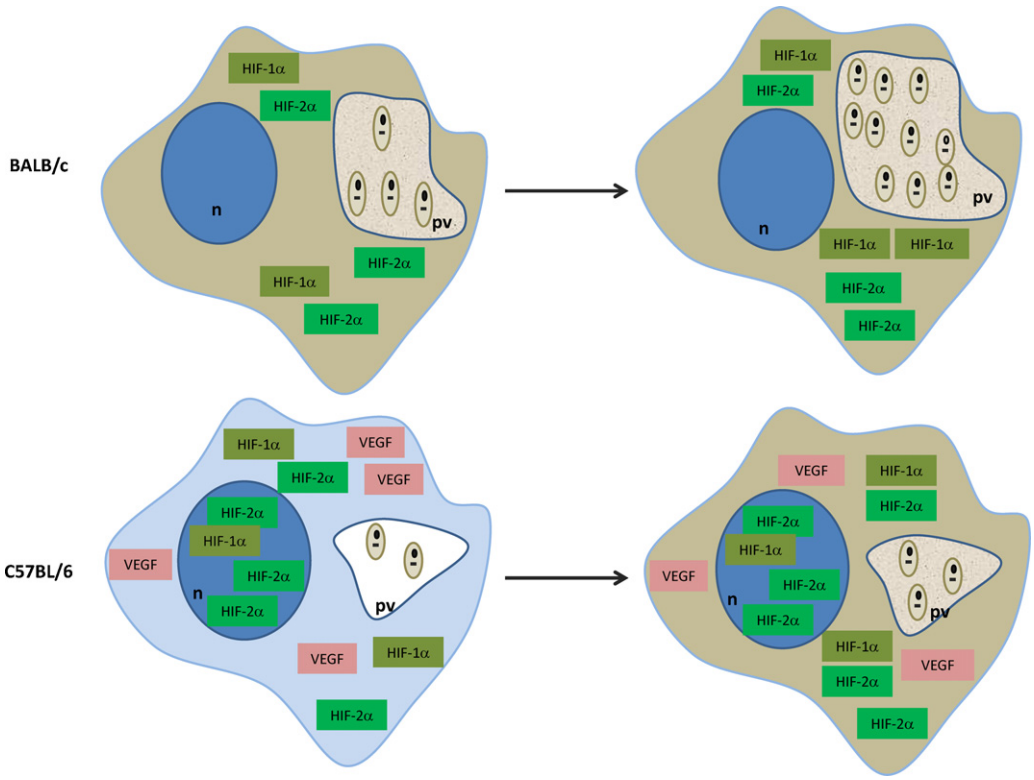

Fig. 6. Scheme illustrating the presence of hypoxia, HIF-1α, HIF-2α and VEGF in *L. amazonensis* infected macrophages present in the lesions of BALB/c and C57BL/6 mice during the experimental period (20 p.i. and 70 p.i.). Blue cell is normoxic and brown cells are hypoxic. Nucleus (n) and parasitophorous vacuole (pv) containing *Leishmania* amastigotes are represented in the figure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

observed in naturally infected humans has lead to the conclusion that they are analogous (Alexander et al., 1999; Nylén and Gautam, 2010). In this study, we confirmed that the BALB/c mice failed to control *L. amazonensis* infection, while the C57BL/6 mice infection leads to controlled progressive lesions. These results may be explained by the experimental findings demonstrating a greater level of nitric oxide occurring in the early stages of infection in the C57BL/6 mice compared with susceptible mice (Giorgio et al., 1998). Despite the immunological paradigm, it was established that there are multiple factors which can determine the resistance and susceptibility to *Leishmania* infection (Bhardwaj et al., 2010). For example, our previous studies indicated that hypoxia may induce functional alterations in macrophages cultured *in vitro* and infected with *L. amazonensis*, including HIF-1α and HIF-2α expression (Degrossoli et al., 2007, 2011). We also demonstrated that infected macrophages accumulate HIF-1α in non-controlled cutaneous lesions in BALB/c mice (Arrais-Silva et al., 2005). In the present study we demonstrated that infected footpads present hypoxic areas in both BALB/c and C57BL/6 mice after 20 and 70 days p.i. respectively. Indeed, hypoxia within lesions is to be expected since, metabolic demand for leukocyte infiltration into infected tissues, parasite proliferation and microcirculatory impairment (Kanan, 1975; Grimaldi and Tesh, 1993; Giorgio et al., 1998; McElrath et al., 1987) should result in increased demand for oxygen (Arrais-Silva et al., 2005). We hypothesized that hypoxia occurs later in the lesions of C57BL/6 mice because the parasite burdens are lower than those of BALB/c mice lesions and consequently the characteristics described above appear later in C57BL/6 mice lesions as shown in the scheme in Fig. 6.

In this study, we also determined the differential phenotype of the susceptible or relatively resistant mice regarding HIF-1α, HIF-2α and VEGF expression. Surprisingly, the results demonstrated that HIF-1α is expressed most of the time during the experimental periods in both mouse strains, and localized predominately in the cytoplasm of infected macrophages and inflammatory cells (Fig. 5). The reasons why HIF-1α, a transcription factor known to accumulate in the nuclei of hypoxic cells, was localized in the cytoplasm of cells within leishmanial lesions are not known. Although HIF-1α has appeared predominantly in nuclei of many mammalian cell types, HIF-1α accumulation was observed in the cytoplasm and nuclei of tumor-associated macrophages and macrophage cell lines under hypoxia (Talks et al., 2000; Crowther et al., 2001; Burke et al., 2002). Previous studies suggested that HIF-1α nuclear accumulation may be impaired by MAPK-dependent phosphorylation (Triantafyllou et al., 2008). Recent reports suggested that reduced importin-α in endothelial cells increases cytoplasmic accumulation of HIF-1α protein (and its absence in the nucleus indicates impaired nuclear transport) and a decrease in transcriptional activation of the VEGF gene (Ahluwalia et al., 2010). Nonetheless, the exact mechanism of decreasing HIF-1α nuclear translocation is still poorly understood. Interestingly, HIF-2α protein in susceptible BALB/c lesions had also been shown to accumulate in the cytoplasm, while in C57BL/6 lesions HIF-2α is translocated to the nucleus and VEGF is produced in footpad tissue by macrophages (Fig. 6). The fact of these small lesions in relatively resistant mice having cells within HIF-2α nuclear sites, strongly suggests that this transcription factor can activate VEGF and cytokine production which are able to induce migration and activation of immunocompetent cells, consequently leading to the control of the infection.

In conclusion, in our study we demonstrated that murine leishmaniasis is a chronic hypoxic disease and it could be useful to analyze hypoxia and hypoxia-related parameters. It may be a valuable experimental tool for examining the nature and order of events

that lead to chronic hypoxia and accumulation of HIFs within macrophages. An increased understanding of the mechanisms of HIF-1α and HIF-2α stabilization will be important for devising new drugs for treatment of chronic infectious and inflammatory diseases and tumor disorders.

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