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ORIGINAL PAPER

Immunohistochemical evidence of stress and inflammatory markers in mouse models of cutaneous leishmaniosis

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Abstract Leishmanioses are chronic parasitic diseases and host responses are associated with pro- or anti-inflammatory cytokines involved, respectively, in the control or exacerbation of infection. The relevance of other inflammatory mediators and stress markers has not been widely studied and there is a need to search for biomarkers to leishmaniasis. In this work, the stress and inflammatory molecules p38 mitogen-activated protein kinase, cyclooxygenase-2, migration inhibitory factor, macrophage inflammatory protein 2, heat shock protein 70 kDa, vascular endothelial factor (VEGF), hypoxia-inducible factors (HIF-1α and HIF-2α), heme oxygenase and galectin-3 expression were assessed immunohistochemically in self-controlled lesions in C57BL/6 mice and severe lesions in Balb/c mice infected with *Leishmania amazonensis*. The results indicated that the majority of molecules were expressed in the cutaneous lesions of both C57BL/6 and Balb/c mice during various phases of infection, suggesting no obvious correlation between the stress and inflammatory molecule expression and the control/exacerbation of leishmanial lesions. However, the cytokine VEGF was only detected in C57BL/6 footpad lesions and small lesions in Balb/c mice treated with antimonial pentavalent. These findings suggest that VEGF expression could be a predictive factor for murine leishmanial control, a hypothesis that should be tested in human leishmaniosis.

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Introduction

Infection of humans and animals with *Leishmania*, an obligate intramacrophage parasite, leads to self-healing, chronic or fatal disease depending on the *Leishmania* species and the host immune status [44, 45]. Mouse models infected with *L. amazonensis*, one of the species involved in localized and diffuse cutaneous leishmaniasis in Brazil, have been an important model for studying pathogen interactions with host immune system [7, 36, 47]. Leishmaniosis has been associated with inflammation because of the involvement of pro- and anti-inflammatory cytokines, such as TNFα, IFNγ, IL1β and IL10, in the exacerbation and control of disease [42, 45]. Numerous reports have described the molecular mechanisms involved in pro- and anti-inflammatory cytokine induction [34, 42]. However, mediators of inflammatory and tissue stress expression appear not to have been studied in situ in leishmanial lesions. Considering the needs to identify biomarkers to leishmaniasis that can be used to diagnose disease, predict clinical outcome, monitor therapeutic responses and immunity [33, 40], we have examined the tissue expression of some inflammatory and stress markers during cutaneous murine leishmaniosis. The predictive or diagnostic values of proteins markers have been suggested for many diseases, for example, hypoxia-inducible factor (HIF-1α) for rectal cancer [43], heme oxygenase (HO-1) for malaria [4] and bladder cancer [41], vascular endothelial growth factor (VEGF) for Sjögren's syndrome [60] and CD31/VEGF for asthma [54]. Thus, similar knowledge could help to

determine the relevance of different host mediators in relation to leishmaniasis control or exacerbation and their predictive value. Recently, our group showed that mice cutaneous lesions present hypoxic areas, i.e., low oxygen tension in the diseased tissue [7, 9]. In addition, infected macrophages from lesions accumulate HIF-1α and HIF-2α [7]. Here, we evaluate the kinetics of some important inflammatory molecules which are detected immunohistochemically in the cellular infiltrate of various inflammatory diseases, in self-controlled lesions in C57BL/6 mice and severe lesions in Balb/c mice infected with *L. amazonensis*, including: p38 mitogen-activated protein kinase (p38 MAPK) and cyclooxygenase-2 (COX-2) in arthritis [15, 22], migration inhibitory factor (MIF) in arthrosclerosis [34], macrophage inflammatory protein 2 (MIP-2) in encephalomyelitis [37], and heat shock protein 70 kDa (HSP70) in colitis [62]. In addition, VEGF, HIF-1α and HIF-2α, HO-1 [25], and galectin-3 (Gal-3) expression [65] were also investigated in mouse models of cutaneous leishmaniosis. We also investigated whether control of *L. amazonensis* infection in mice treated with pentavalent antimonial, the standard anti-leishmanial agent [28] could be associated with differential inflammatory and stress molecules expression in lesion tissue.

Materials and methods

Animals

Female BALB/c and C57BL/6 mice (6 weeks old) were obtained from the Animal Center of Campinas State University (UNICAMP), Campinas, SP, Brazil. The experimental protocols were approved by the Institute of Biology, Campinas State University Ethical Committee for Animal Research.

L. amazonensis infection, evaluation and mice treatment

L. amazonensis (MHOM/BR/73/M2269) was maintained by regular passage in BALB/c mice, as described previously [12]. In the experiments, mice were injected subcutaneously in footpad with 10^5 amastigotes. The course of the infection was monitored by measuring the increase in footpad thickness compared with the contra lateral uninfected footpad, with a dial caliper [30]. Alternatively, mice were infected with 10^5 amastigotes suspended in 20 µl of sterile saline by subcutaneous injection into the shaved rump. Lesions were measured at weekly intervals in two perpendicular diameters with a dial caliper [13, 14] and were expressed as the dimensions of the nodule base (width by length). To estimate parasite burden in the lesions, the mice were killed at designated time points, the lesioned tissues were removed and weighed and amastigotes were recovered from the lesions and counted [9]. Three independent experiments involving 8–10 mice each were performed to evaluate infection and conduct the immunohistochemical study. Mice were treated with pentavalent antimonial (*N*-methyl glucamine antimoniate; Aventis, São Paulo, Brazil), the standard anti-leishmanial agent at 27 mg Sb^{+5}/kg/day injected intraperitoneally, once a day for 20 days after *L. amazonensis* inoculation [13]. The control group was left untreated. Two independent experiments involving 8 mice each were performed to evaluate infection and conduct a comparative immunohistochemical study.

Immunochemistry

As described previously [7], the lesion tissues from mice killed at designated time points were fixed for 24 h with paraformaldehyde and embedded in paraffin blocks. Tissue sections (5 µm) 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, OH, USA) or normal mouse serum for 30 min. Tissue sections were then incubated overnight in a humid chamber at 4 °C with the following primary antibodies: goat polyclonal anti-COX-2 (Abcam, MA, USA), rabbit polyclonal anti-Gal 3 (Abcam), rabbit polyclonal anti-HIF-1α (Santa Cruz Biotechnology, CA, USA), rabbit polyclonal anti-HIF-2α (a gift from Dr. Darren Richard), rabbit polyclonal anti-HSP70 (Abcam), rabbit polyclonal anti-MIF (Santa Cruz), rabbit polyclonal anti-MIP-2 (Santa Cruz), rabbit polyclonal anti-HO-1 (Abcam), rabbit polyclonal anti-p38 MAPK (Abcam) or rabbit polyclonal anti-VEGF (Calbiochem, CA, USA and Chemicon, Temecula, CA, USA). Control sections were incubated with normal rabbit serum, rabbit polyclonal antibody or mouse normal serum as primary antibody. Primary antibodies were detected by species-specific secondary antibodies: goat polyclonal anti-rabbit conjugated with peroxidase (Sigma-Aldrich, MA, USA) or rabbit polyclonal anti-goat conjugated with peroxidase (Sigma-Aldrich), incubated during 45 min in a humid chamber. A control section included the incubation with secondary antibodies in the absence of primary antibodies. Primary and secondary antibodies did not react with amastigotes isolated from the lesions. Tissue sections were visualized with a peroxidase substrate solution containing 3,3-diaminobenzidine and hydrogen peroxide [7, 8] and counterstained with hematoxylin, dehydrated in graded alcohol solutions and mounted in cytoseal-60 mounting medium (Sigma). The extent and intensity of the expression of each marker were

analyzed, and the intensity of staining was scored as positive (strong to weak staining) and negative (absence of staining). The percentage of cells that exhibited positive staining was calculated based on the evaluation of at least 20 random fields. The images were captured with a digital imaging system consisting of a light microscope (Eclipse E800, Nikon), a Cool Snap-Pro Color camera (Media Cybernetics) and capture software Image-Pro plus (Media Cybernetics).

Pimonidazole

The mice were administered intraperitoneally with pimonidazole hydrochloride (Hypoxyprobe-1, Chemicon) dissolved in sterile, filtered physiological saline at 60 mg/kg body weight. After 90 min, the lesion tissue was fixed with 4 % paraformaldehyde and embedded in paraffin, followed by immunohistological detection of pimonidazole protein adducts, following the manufacturer's recommendations and as described previously [7, 8].

Results

Evolution of *L. amazonensis* infection

Macroscopic analysis of Balb/c and C57BL/6 mice inoculated with *L. amazonensis* in the hind footpad showed marked differences between two inbred mice. C57BL/6 mice presented small and non-ulcerated lesions during the course of infection, while expanding ulcerous lesions were observed in Balb/c mice (Fig. 1). In the rump infection model, Balb/c mice developed a slowly growing ulcerated round lesion with raised borders (Fig. 1a, h, i, j), the choice of this mouse model was due to its development of cutaneous lesion closely corresponding to the clinical setting [13, 40]; C57BL/6 mice did not develop any lesion at the rump inoculation site or elsewhere (data not shown) and consequently were not evaluated in this study. The stages of leishmanial infection in mice were divided based on time after parasite inoculation and skin lesion characteristics: initial stage—small, palpable lesion observed in Balb/c foodpad model at 2 dpi (days post-inoculation) and in Balb/c rump model at 40 dpi; intermediate stage—lesion of moderate size observed in Balb/c footpad model at 20 dpi and Balb/c rump model at 70 dpi; and, late stage—ulcerated and large lesion observed in Balb/c foodpad model at 70 dpi and Balb/c rump model at 110 dpi. In the case of C57BL/6 footpad model, small lesions were observed throughout the entire experiment. The stages of infection: initial (2 dpi), intermediate (20 dpi) and late (70 dpi), defined for Balb/c footpad model mice was also used for the C57BL/6 footpad model.

All lesions induced by *L. amazonensis* showed inflammatory cell infiltrate (Fig. 2). A quantitative evaluation of the cellular profile in the lesions is shown in Table 1. At the initial phase (2 dpi), a small number of infiltrating cells located predominantly at the dermis were observed in Balb/c and C57BL/6 footpad lesions. Macrophages become the predominant infiltrating cell type and polymorphonuclear cells (PMN) and lymphocytes appears in small numbers in Balb/footpad lesions at intermediate and late stages of infection (20 and 70 dpi); in these lesions many parasitized and vacuolated macrophages were observed (Fig. 2; Table 1). In C57BL/6 footpad lesions there were fewer infiltrating cells than in Balb/c footpad lesions, a macrophage influx peaked at 20 dpi and few parasitized cells were observed during the course of infection; PMN were the predominant inflammatory cells in lesions of late phase of infection (Fig. 2; Table 1). The Balb/c rump lesions showed intense cellular infiltrate and, although an accumulation of macrophages was observed, it was less than that observed in the footpad model; moreover, PMN inflammatory cells also detected in lesions by the late stage of infection (110 dpi) (Fig. 2). Numerous vacuolated parasitized macrophages were observed in the lesions at all time points (Fig. 2). These data (Figs. 1, 2; Table 1) confirm previous findings that Balb/c mice display a susceptible phenotype, C57BL/6 mice control *L. amazonensis* infection and parasite inoculation triggers a local influx of inflammatory cells.

Immunohistochemical studies

Immunohistochemical studies analyzed whether L. amazonensis affected the pattern of inflammatory, stress and hypoxia-related proteins VEGF, MIF, p38 MAPK, COX-2, MIP-2, HSP70, HIF-1α, HIF-2α, HO-1 and Gal-3. Table 2 resumes the data obtained for protein immunostaining related to hypoxia and Fig. 3 shows some examples of typical positive and negative immunostainings. Antibodies against pimonidazole, a surrogate marker of hypoxia, which is reduced, protein bound and accumulated in tissues in low oxygen tension [51], HIF-1α, HIF-2α, VEGF and HO-1 were consistently non-reactive in normal mice footpad and rump tissue (data not shown). There was no evidence of hypoxia in footpad lesions from Balb/c and C57BL/6 footpad lesions in the initial phase of infection. C57BL/6 footpad lesions only became hypoxic by the late stage of infection, while hypoxia began earlier in Balb/c footpad lesions. Hypoxia was detected in Balb/c rump lesions throughout the course of infection. HIF-1α and HIF-2α were expressed in the intermediate and late stages of infection in C57BL/6 and Balb/c footpad lesions, in contrast, HIF-1α and HIF-2α were expressed only in the late stage of Balb/c rump lesions. No evidence of VEGF

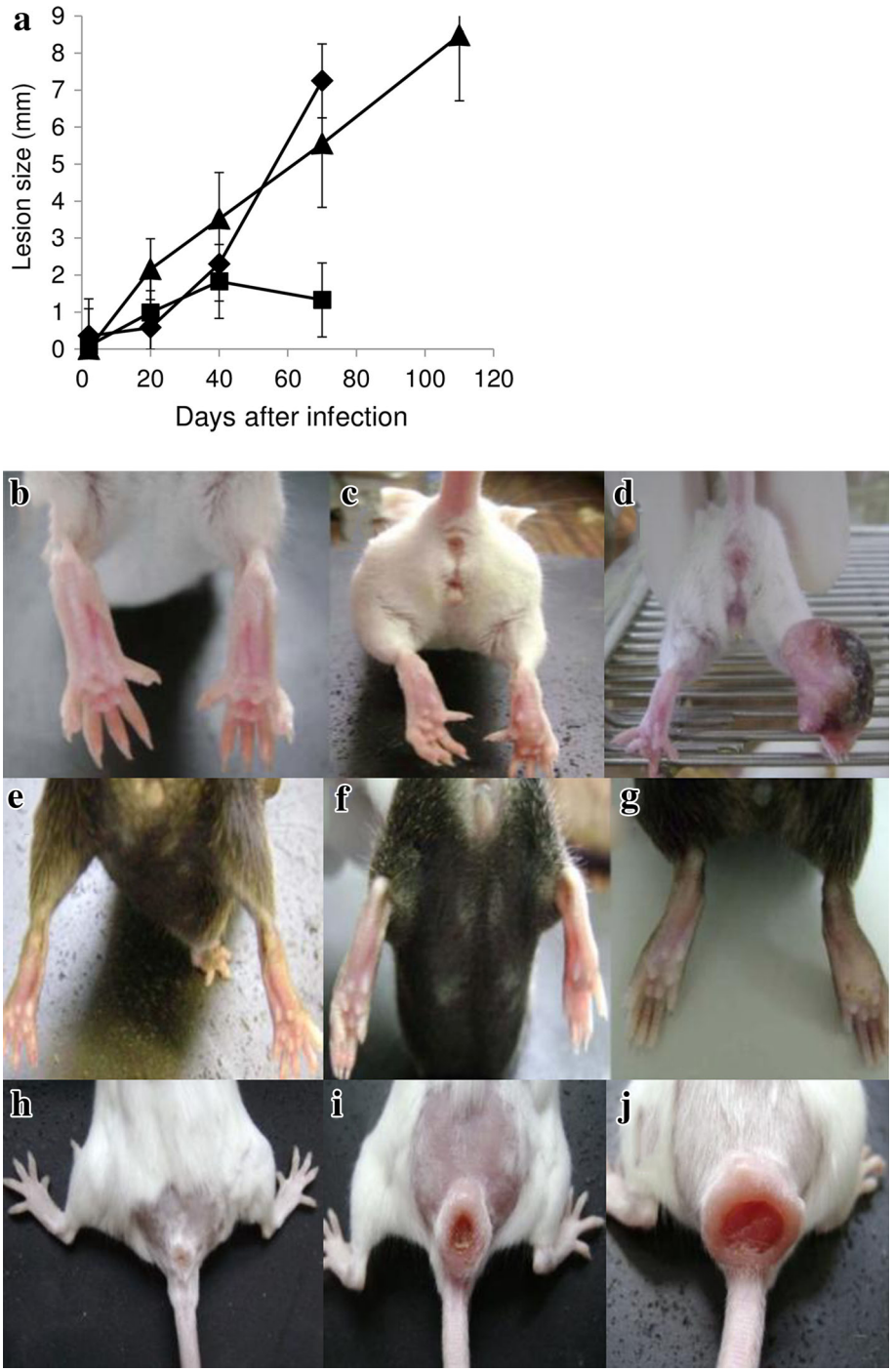

Fig. 1 Evaluation of lesions on the course of *L. amazonensis* infection in mice. **a** Lesion size of Balb/c (*filled diamond*) and C57BL/6 (*filled square*) injected subcutaneously in footpad with 10^5 amastigotes, and Balb/c mice (*filled triangle*) infected subcutaneously in shaved rump with 10^5 amastigotes. **b**, **c**, **d** Balb/c footpad lesions at 2, 20 and 70 dpi. **e**, **f**, **g** C57BL/6 footpad lesions at 2, 20, 70 dpi. **h**, **i**, **j** Balb/c rump lesions at 40, 70, 110 dpi

expression was verified in Balb/c footpad and rump lesions at any time postinfection, while C57BL/6 footpad lesions showed VEGF expression in the intermediate and late stages of infection (Table 2; Fig. 3). The staining for VEGF observed in these lesions was detected in the extracellular spaces, as well as in macrophages (Fig. 3). HO-1 was detected in C57BL/6 footpad lesions in the intermediate and late phases of infection and Balb/c footpad lesions only in the late stage of infection, when its expression in inflammatory cells and macrophages showed diffuse cytoplasmic staining (Fig. 3e). Examination of Balb/c rump lesions indicated HO-1 staining pattern similar to that observed in Balb/c footpad lesions.

Table 3 summarizes the data for immunostaining for MAPK p38, COX-2, HSP70, MIP-2 and gal-3 expression in mouse lesions. p38 MAPK was positive in both Balb/c

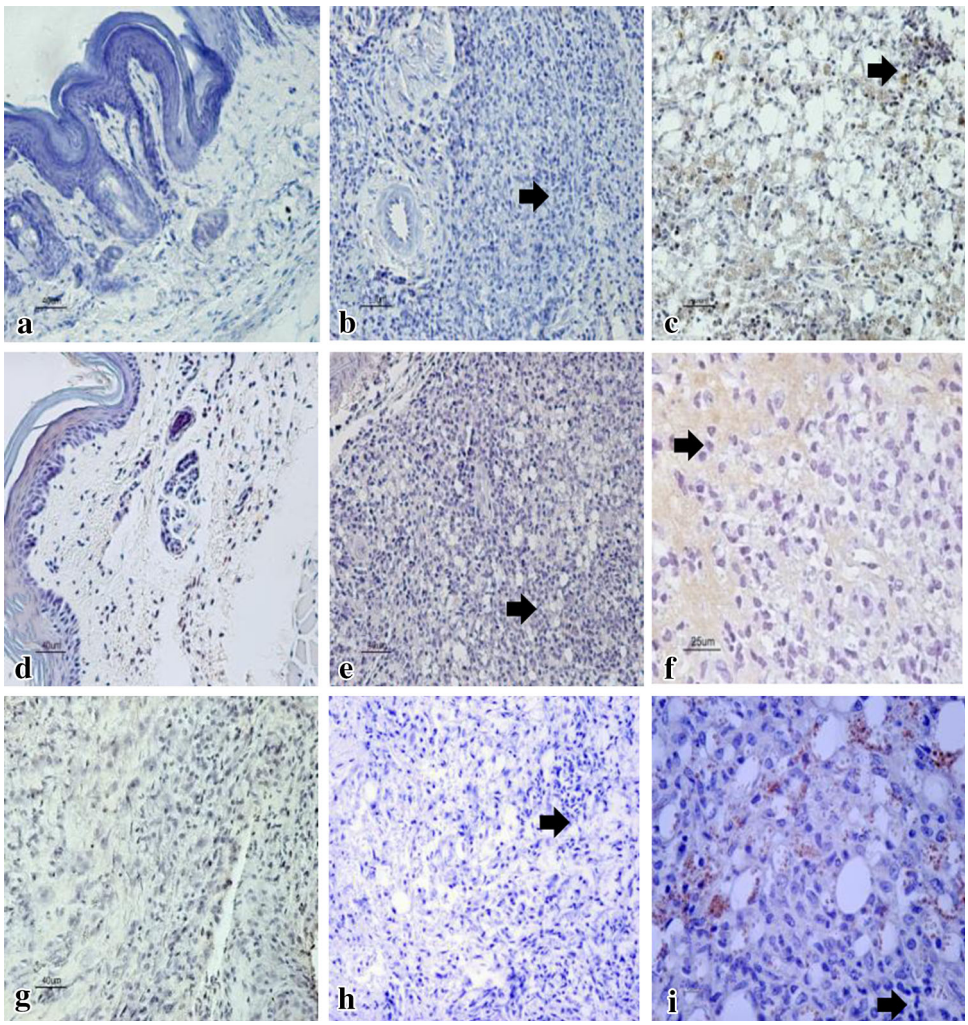

Fig. 2 Evaluation of histological patterns of *L. amazonensis*-infected mouse lesions. **a** Balb/c footpad lesion at 2 dpi (normal epidermis and dermis and a diffuse small cell infiltrate). **b** Balb/c footpad lesion at 20 dpi (mixed cell infiltrate). **c** Balb/c footpad lesion at 70 dpi (infected macrophages replaced tissue). **d** C57BL/6 footpad lesion at 2 dpi (diffuse small cell infiltrate). **e** C57BL/6 footpad lesion at 20 dpi (infiltration of macrophages). **f** C57BL/6 footpad lesion at 70 dpi (cell infiltrate). **g** Balb/c rump lesion at 40 dpi (normal epidermis and dermis, and a diffuse small cell infiltrate). **h** Balb/c rump lesion at 70 dpi (mixed cell infiltrate). **i** Balb/c rump lesion at 110 dpi (mixed cellular infiltrate and many infected macrophages). The *arrows* indicate PMN cells

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

Mouse strain (site)	dpi[a]	Macrophages[b]	Other cells[c]
Balb/c (footpad)	2	47 (≤10)	53
	20	58 (10)	42
	70	90 (≥60)	10
C57Bl/6 (footpad)	2	69 (≤1)	31
	20	81 (≤10)	19
	70	35 (≤30)	65
Balb/c (rump)	40	17 (≤20)	83
	70	33 (≤40)	67
	110	46 (≤70)	54

The lesions were stained with hematoxylin and cells were counted in 20–10 random fields

[a] Days post-inoculation

[b] The % of total macrophages (number in parenthesis indicates % of infected macrophages)

[c] The % of inflammatory cells: PMN cells and lymphocytes

footpad and rump lesions and in C57BL/6 footpad lesions only in the late stages of infection. It was detected in areas containing macrophages. MIF was detected in C57BL/6 footpad lesions throughout the course of infection, but not in the initial stage of infection in Balb/c footpad and rump lesions (Table 3). MIF was expressed in inflammatory cells in skin lesions and its immunoreactivity was also detected in epidermal and dermal layers of skin from normal C57BL/6 mice. COX-2 is expressed continuously in all skin lesions (rump and footpad) throughout the course of infection; staining for COX-2 was detected in infected macrophages and other infiltrating inflammatory cells. In normal mice footpad tissue, no COX-2 expression was observed, but COX-2 positive cells were observed in the glandular structure of normal skin rump (data not shown). HSP70 expression was detected in Balb/c rump lesions throughout the course of infection and Balb/c and C57BL/6 footpad lesions in the intermediate and final stages of infection. HSP70 immunoreactivity was observed in the

Table 2 Pimonidazole, HIF-1α, HIF-2α, VEGF and HO-1 immunostaining in lesions of mice infected with *L. amazonensis*

Mouse strain (site)	dpi[a]	Pimonidazole	HIF-1α	HIF-2α	VEGF	HO-1
Balb/c (footpad)	0[b]	0	0	0	0	0
	2	0	0	0	0	0
	20	20 ± 2	15 ± 3	24 ± 8	0	0
	70	95 ± 1	60 ± 4	75 ± 2	0	78 ± 8
C57Bl/6 (footpad)	0	0	0	0	0	0
	2	0	0	0	0	0
	20	0	55 ± 8	65 ± 1	30 ± 6	20 ± 6
	70	40 ± 5	82 ± 1	85 ± 2	10 ± 8	82 ± 2
Balb/c (rump)	0	0	0	0	0	0
	40	48 ± 1	0	0	0	0
	70	86 ± 1	0	0	0	0
	110	95 ± 2	38 ± 2	15 ± 4	0	80 ± 2

The % of cells ± SD that exhibited positive staining was calculated as detailed in "Materials and methods"

[a] Days post-inoculation

[b] Uninfected normal mice

majority of inflammatory cells infiltrating skin lesions. MIP-2 was expressed in all mice lesions (footpad and rump) throughout the course of infection; staining for MIP-2 was also observed in the glandular structures of rump lesions and macrophages. Gal3 was detected immunohistochemically in epidermal cells of normal Balb/c and C57BL/6 footpad.

The next question was whether expression of these markers changes in susceptible Balb/c mice treated with the standard chemotherapy. As depicted in Fig. 4, mice treated with pentavalent antimonial resulted in a marked diminution of footpad lesion size and the average inhibition of parasite burden was 91.4 % (1.5×10^7 mean value of parasites per lesion from treated mice versus 17.5×10^7 mean value of parasites per lesion from untreated mice) at 50 dpi (Fig. 4b). Among the seven markers tested (pimonidazole, HIF-1α, HIF-2α VEGF, HO-1, p38 MAPK and MIF), only VEGF was differently expressed between the footpad lesions of treated and untreated mice (Table 4), such that positive VEGF immunostaining was detected in the lesions of pentavalent antimonial-treated Balb/c mice.

Discussion

This study investigated the differential phenotype of mice susceptible and relatively resistant to *L. amazonensis* regarding inflammatory and tissue stress mediator expression. A schematic representation of inflammatory and stress molecules expressed in tissue lesions of mice is shown in Fig. 5. The lectin Gal-3 was expressed in cells of lesion tissues from each mouse in all phases of infection and also in foodpad tissue of normal mice. In fact, Gal3 is widely expressed in epithelial and immune cells and it has been known to be upregulated in numerous tumors and in chronic inflammatory diseases such as rheumatoid arthritis and asthma [29]. However, no difference was observed in Gal-3 expression between lesions from Balb/c and C57BL/6 mice. Gal3 expression remained unaltered during infection with *L. amazonensis*.

Analysis of the results showed that MIP-2 is expressed in cells from all skin lesions of mice throughout the course of infection. MIP-2 is produced by macrophages and epithelial cells after stimulation with lipopolysaccharides (LPS) or inflammatory [46] and chemotactic for neutrophils [31]. Previous studies have shown that *L. major* inoculated within a murine air pouch system induces MIP-2 gene expression in the exudate cells [39]; the authors suggested that MIP-2 plays a role in neutrophil accumulation in response to the parasite. This is in agreement with previous studies showing a prolonged presence of PMN cells mainly neutrophils and eosinophils [5, 40, 56] and our results showing a prolonged presence of PMN cells in leishmanial lesions and MIP-2 protein expression during the infection.

COX-2 is expressed continuously in all skin lesions of mice throughout the course of infection in macrophages and other infiltrating inflammatory cells. COX-2 is the inducible isoform of COX pro-inflammatory enzyme [53] associated with the production of proteinoids under inflammatory conditions [64], such that it is expressed in only a few normal tissues and upregulated in inflamed tissues. A previous study showed that *L. mexicana* prolonged the induction of COX-2 in macrophages stimulated with LPS [59]. Thus, its expression was expected, although no differences were observed between lesions from Balb/c and C57BL/6 mice.

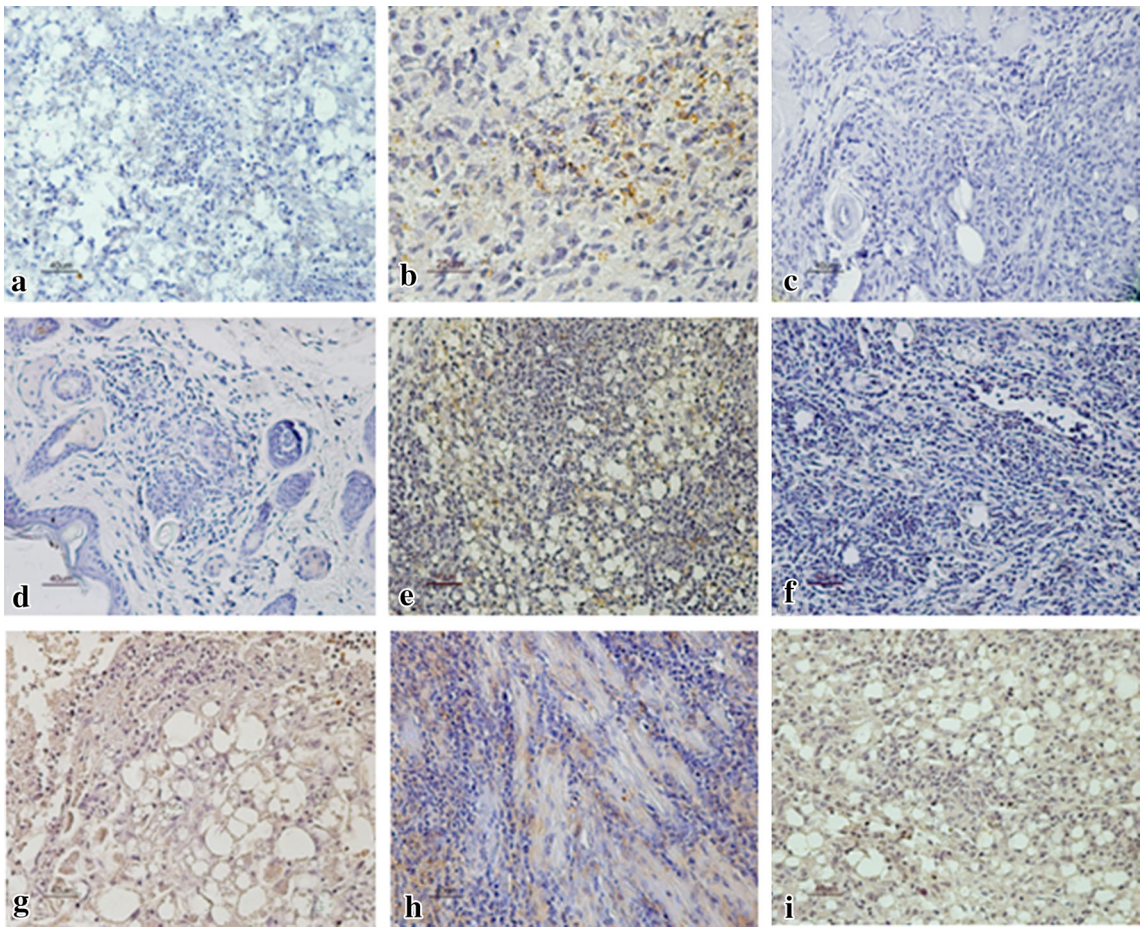

Fig. 3 Representative photographs of immunohistochemical staining of mouse lesions. **a** Negative immunostaining for VEGF in Balb/c footpad lesion at 70 dpi. **b** Positive immunostaining for VEGF in B75BL/6 footpad lesion at 70 dpi. **c** Negative immunostaining for VEGF in Balb/c rump lesion at 110 dpi. **d** Negative immunostaining for HO-1 in Balb/c footpad lesion at 20 dpi. **e** Positive immunostaining for HO-1 in C57BL/6 footpad lesion at 20 dpi. **f** Negative immunostaining for HO-1 in Balb/c rump lesion at 70 dpi. **g** Positive immunostaining for p38 MAPk in Balb/c footpad lesion at 70 dpi. **h** Positive immunostaining for p38 MAPk in B75BL/6 footpad lesion at 70 dpi. **i** Positive immunostaining for p38 MAPk in Balb/c rump lesion at 110 dpi

Hsp70 is one of the heat shock glycoproteins that stabilize intracellular processes of cells under temperature changes or stress, its expression has been reported in various models of injury, including skin excision, laser and ultraviolet irradiation and vitiligo [1, 57]. Thus, it is not surprising that cells within leishmanial lesions expressed HSP70. In this work, HSP70 expression was detected earlier in rump lesions than in footpad lesions. This could be explained by the fact that footpad skin has an epithelium and a thicker dermis and the connective tissue is denser than that of the hairy skin (back rump regions, for example) [27], reflecting in local differences in the skin temperature (28–29 °C on the footpad and 34 °C on the back), and stress responses.

This study demonstrated MIF expression in cell layers of the epidermis of the footpad of normal mice, as previously described [2], and in inflammatory cells of the majority of macrophages from rump and footpad lesions. MIF is an immune neuroendocrine mediator with pleiotropic effect but it is also a key inflammatory cytokine in many chronic diseases [17, 58]. Previous studies showed that elevated plasma levels of MIF is correlated with the chronic immune hyperactivation involved in T cell depletion observed in visceral leishmaniosis patients [58], but in this study, MIF is not associated with controlled or uncontrolled leishmanial cutaneous lesions.

The expression of p38 MAPK occurred in Balb/c and C57BL/6 footpad lesions and rump lesions only in the late phases of infection. p38 MAPK is a member of the MAPK-activated protein kinase complex activated by stress signals [20], and previous in vitro studies have indicated that infection by *L. donovani* induces macrophage phosphotyrosine phosphatases that attenuated MAPK signaling [32]. The authors suggest that *Leishmania* modulates innate immune response by inhibiting p38 MAPK activation. Our study suggests no clear

Table 3 p38 MAPK, MIF, COX-2, HSP70, Gal-3 and MIP-2 immunostaining in lesions of mice infected with *L. amazonensis*

Mouse strain (site)	dpi[a]	p38 MAPK	MIF	COX-2	HSP70	Gal-3	MIP-2
Balb/c (footpad)	0[b]	0	0	0	0	8 ± 4	0
	2	0	0	8 ± 3	0	10 ± 3	32 ± 5
	20	0	46 ± 6	43 ± 7	69 ± 1	88 ± 6	71 ± 1
	70	60 ± 4	88 ± 5	81 ± 2	89 ± 5	91 ± 3	78 ± 5
C57Bl/6 (footpad)	0	0	25 ± 4	0	0	15 ± 1	0
	2	0	15 ± 7	10 ± 3	0	17 ± 1	42 ± 2
	20	0	89 ± 4	76 ± 6	36 ± 4	43 ± 5	86 ± 3
	70	56 ± 6	96 ± 1	90 ± 4	78 ± 6	56 ± 2	81 ± 6
Balb/c (rump)	0	0	0	8 ± 3	0	0	0
	40	0	0	28 ± 8	6 ± 1	20 ± 4	43 ± 3
	70	0	21 ± 4	75 ± 6	10 ± 3	89 ± 3	51 ± 1
	110	78 ± 6	43 ± 8	90 ± 4	68 ± 4	82 ± 5	78 ± 6

The % of cells ± SD that exhibited positive staining was calculated as detailed in "Materials and methods"

[a] Days post-inoculation

[b] Uninfected normal mice

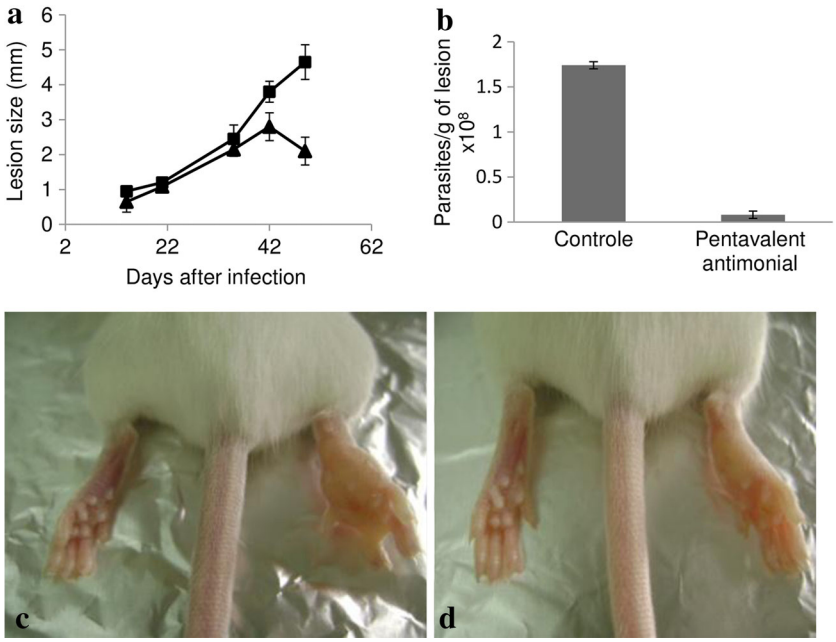

Fig. 4 Evaluation of lesions and stress markers on the course of *L. amazonensis* infection in mice treated with pentavalent antimonial. Balb/c mice were injected subcutaneously in footpad with 10^5 amastigotes and treated with pentavalent antimonial (*filled square*) during 20 days, or left untreated (*filled diamond*). **a** Lesion size. **b** Parasite numbers were evaluated after 50 dpi. **c** Untreated mice footpads. **d** Pentavalent antimonial-treated mice footpads

association between p38 MAPK and controlled and uncontrolled leishmanial lesions.

HO-1 is also a stress-responsive enzyme, and induction of HO-1 results in the catabolism of prooxidant heme to biliverdin and bilirubin, which are potent antioxidants [25]. Many anti-inflammatory properties are attributed to HO-1 and the beneficial effects of HO-1 have been reported in various models of inflammation, including pleural inflammation, renal injury and small bowel ischemia [11, 25, 38, 48, 63] showed that *L. pifanoi* and *L. chagasi* infections trigger an increase in HO-1 levels in cultures of macrophages and serum from patients with visceral leishmaniasis. In our study, HO-1 was detected in the footpad and rump models. Interestingly, HO-1 appeared earlier in C57BL/6 mice footpad lesions than in Balb/c footpad and rump lesions. Thus, it can be hypothesized that the presence of HO-1 during the early phase of infection in C57BL/6 lesions protects host tissue against an excessive inflammatory response. In fact, C57BL/6 mice develop small inflammatory lesions throughout the period of infection. In the later phases of infection, particularly in susceptible Balb/c mice, HO-1 can play another role that involves decreasing the host ability to eliminate parasites [38].

Table 4 Pimonidazole, HIF-1α, HIF-2α, VEGF, HO-1, p38 MAPk and MIF immunostaining in lesions of mice infected with *L. amazonensis* and treated with pentavalent antimonial

Markers	Balb/c[a] (control)	Balb/c (pentavalent antimonial)
Pimonidazole	93 ± 3[b]	76 ± 7
HIF-1α	25 ± 2	78 ± 4
HIF-2α	72 ± 3	68 ± 4
VEGF	0	38 ± 6
HO-1	71 ± 3	86 ± 6
p38 MAPk	61 ± 6	93 ± 4
MIF	70 ± 7	90 ± 5

[a] Balb/c mice were injected subcutaneously in footpad with 10^5 amastigotes and treated with pentavalent antimonial during 20 days, or left untreated (control); examination of lesions at 50 dpi

[b] The % of cells ± SD that exhibited positive staining was calculated as detailed in "Materials and methods"

This study confirmed previous data that hypoxic areas occur in Balb/c footpad lesions in the intermediate stage of infection and in C57BL/6 footpad lesions in the late stage of infection [7], and extended these observations by showing that Balb/c rump lesions contain hypoxic areas throughout course of infection. Furthermore, the expression of the two transcription factors HIF-1α and HIF-2α coincides temporally with the appearance of hypoxic areas in Balb/c footpad lesions and occurs later than the appearance of hypoxic areas in rump lesion. More interestingly, HIF-1α and HIF-2α expressions occur earlier than the appearance of hypoxic areas in C57BL/6 footpad lesions, suggesting activation of HIFs independent of hypoxia. In fact, we and others have previously noted that HIFs expression could be independent of hypoxic stimuli [23, 24, 55]. In an in vitro system, *L. amazonensis*-infected macrophages express HIFs, although pimonidazole adducts are not found intracellularly; i.e., low oxygen tension (1–2 %) was not detected in cell cultures [23]. It should be noted that HIF-1α is overexpressed in various tumor cells by hypoxia-independent mechanisms [35]. Thus, others factors present in tissue lesions could be involved in stabilizing HIF expression during different phases of infection, and further studies are required to elucidate these points.

The majority of the stress and inflammatory molecules studied here were expressed during the various phases of

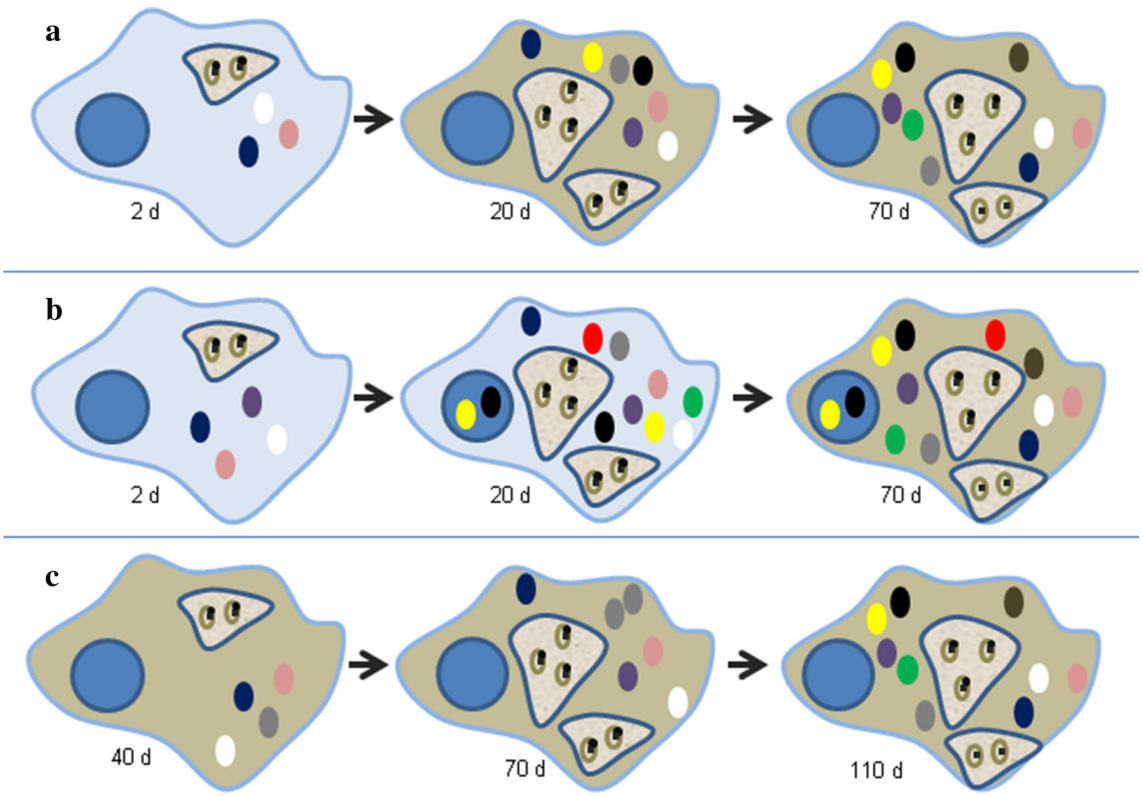

Fig. 5 Scheme illustrating the presence of inflammatory and stress molecules in lesions on the course of *L. amazonensis* infection in mice. **a** Balb/c footpad lesions. **b** C57BL/6 footpad lesions. **c** Balb/c rump lesions. At 2, 20 and 70 dpi for footpad lesions and 40, 70 and 110 dpi for rump lesions. Hypoxia (*brown* background), HIF-1α (*yellow circle*), HIF-2α (*black circle*), VEGF (*red circle*), HO-1 (*green circle*), p38 MAPK (*brown circle*), MIF (*purple circle*), COX-2 (*blue circle*), MIP-2 (*pink circle*), HSP70 (*gray circle*), Gal-3 (*white circle*)

infection in Balb/c and C57BL/6 footpad lesions and Balb/c rump lesions, suggesting no obvious correlation between the expression of stress and inflammatory molecules and the control/exacerbation of leishmanial lesions. However, in the case of VEGF, it is tempting to infer a correlation between VEGF expression and leishmanial control, since its expression was detected only in C57BL/6 footpad lesions. This is also supported by our observations that VEGF is detected in lesions from Balb/c mice infected with *L. amazonensis* and treated with antimonial pentavalent. This treatment ameliorated the course of disease by reducing lesion size and parasite load, although no complete cure was observed [13]. VEGF is an angiogenic factor produced and secreted by a number of cell types, including muscle cells, fibroblast and macrophages [6, 21, 52], and induces endothelial cell proliferation and stimulates capillary formation in models of angiogenesis, such as embryonic development, wound healing, inflammatory diseases and tumors [18, 21, 61]. VEGF can also induce monocyte chemotaxis [19]. At least two questions can be raised concerning the VEGF expression in controlled lesions from C57BL/6 mice infected with *L. amazonensis*. First, what stimuli are involved in VEGF production? Indeed many stimuli for VEGF production have been identified which could be involved in leishmanial infection, including hypoxia, HIFs, cytokines and nitric oxide (NO) [18, 50, 66]. Of relevance to this work is the cytokine interferon-γ (IFN-γ) [52], which is associated with host resistance to *Leishmania* [3], and produced by C57BL/6 mice infected with *L. amazonensis* [10, 49]. In addition, endogenous NO enhances VEGF synthesis in in vitro models, including macrophages [52, 66], and is associated with control of leishmaniasis, since high levels of NO are known to be leishmanicidal and are continuously produced by C57BL/6 mice infected with *L. amazonensis* [30]. Future studies are required to determine whether these factors (hypoxia, HIFs, NO and/or IFN-γ) could be linked to VEGF expression in C57BL/6 mice lesions. Our study also raises the question of the functional role of VEGF in controlled cutaneous lesions. The most obvious answer is that VEGF facilitates the recruitment of immune effector cells [67] to the injury-infected tissue. However, VEGF has also been described as cytoprotectant against oxidative stress [12, 16, 26], and could play a protective role in the preservation of the integrity of vasculature and tissue function during infection. Again, further study is required to determine the functional role of VEGF and test VEGF as predictor factor related to resistant/susceptibility to the disease, and chemotherapy response in murine infection models developed with other *Leishmania* species which mimic the pathological and immunological responses observed in humans. In summary, the immunohistochemical evidence obtained in this study indicates that the VEGF expression may be a predictive factor for murine leishmanial control, a hypothesis that should be tested in human leishmaniosis. In the future, integration of the protein markers, identified using experimental models, into medical practice will be crucial for treatment and prevention of this neglected parasitic disease.

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