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Expression of hypoxia-inducible factor 1α in mononuclear phagocytes infected with *Leishmania amazonensis*

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Abstract

Increasing evidence indicates that hypoxia-inducible factor 1α (HIF-1α) can be upregulated in different cell types by nonhypoxic stimuli such as growth factors, cytokines, nitric oxide, lipopolysaccharides and a range of infectious microorganisms. In this study, the ability of the following mononuclear phagocytes to express HIF-1α is reported: mouse macrophages (mMΦ), human macrophages (hMΦ) and human dendritic cells (DC), parasitized in vitro with *Leishmania amazonensis*; as assessed by immunofluorescence microscopy. A logical explanation for HIF-1α expression might be that the mononuclear phagocytes became hypoxic after *L. amazonensis* infection. Using the hypoxia marker pimonidazole, observation revealed that *L. amazonensis*-infected cells were not hypoxic. In addition, experiments using a HIF-1α inhibitor, $CdCl_2$, to treat *L. amazonensis*-infected macrophage cultures showed reduced parasite survival. These studies indicated that HIF-1α could play a role in adaptative and immune responses of mononuclear phagocytes presenting infection by the parasite *L. amazonensis*.

Keywords: Hypoxia-inducible factor 1α; Mononuclear phagocytes; *Leishmania amazonensis*

1. Introduction

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcriptional protein consisting of HIF-1α and HIF-1β components [1,2]. Whereas HIF-1β is constitutively expressed in all cells, HIF-1α has been shown to stabilize and accumulate in cells during hypoxia, mainly through inhibition of its degradation by the ubiquitin–proteasome system [2,3]. The expression of different genes is controlled at a transcriptional level by HIF-1α, including erythropoietin, vascular endothelial growth factor, glucose transporters and glycolytic enzymes [2]. Recently, our group showed that HIF-1α is expressed in cutaneous lesions of mice infected with *Leishmania amazonensis*, a parasite of mononuclear phagocytes and one of the causative agents of cutaneous and diffuse cutaneous leishmaniasis in the Americas [4,5]. Immunohistochemical analyses demonstrated HIF-1α positive infected macrophages throughout the lesions suggesting a hypoxic intralesional microenvironment [5]. Although the current paradigm supports HIF-1α as a regulator of the genetic response to hypoxia [2,6–9], it has been shown that a number of stimuli, such as iron chelator, growth factors and hormones, increase HIF-1α in a normoxic condition [10–13]. In addition, demonstrations that HIF-1α can also be activated in selected cell lines infected with *Bartonella henselae*, *Streptococcus*, *Staphylococcus aureus*, *Pseudomonas*, *Salmonella*, *Chlamydia pneumoniae*, Epstein Barr virus and the eukaryotic parasite *Toxoplasma gondii* under normoxic conditions [14–19], led us to examine the expression of HIF-1α and pimonidazole adduct formation, a chemical marker for hypoxia, in macrophages (MΦ) and dendritic cells (DC) infected with *L. amazonensis*.

2. Materials and methods

2.1. Parasites

L. amazonensis (MHOM/BR/73/M2269) promastigotes were cultured at 28 °C in RPMI 1640 medium (Nutricell, Campinas, SP, Brazil) supplemented with 25 μg/mL gentamicin, 2 mM L-

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glutamine, 100 mM HEPES (Sigma, St. Louis, MO), and 10% fetal calf serum (FCS) (Nutricell), pH 7.4. *L. amazonensis* amastigotes were isolated from active skin lesions of BALB/c mice, as previously described [20].

2.2. Cell culture

Primary mouse macrophages (mMΦ) were obtained from normal BALB/c mice by peritoneal lavage, as previously described [21]. The cells were cultured with complete RPMI 1640 medium in 16-well slide-chambers (Nunc Inc., Naperville, IL) (2×10^5 mMΦ/well) at 37 °C in 21% O_2, 5% CO_2 and balanced N_2. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by centrifugation over Ficoll-hypaque 1.077 (Sigma) as previously described [22]. Monocytes were cultured for 7 days with Iscove's medium (Sigma) supplemented with 25 μg/mL gentamicin and 10% FCS, pH 7.4, in 16-well slide-chambers (2×10^5 MΦ/well), to achieve differentiation into macrophages (hMΦ). The generation of DC from PBMC drawn from healthy donors was realized, as previously described [23]. DC were cultured for 7 days in complete Iscove's medium supplemented with interleukin 4 (250 ng/mL) and granulocyte macrophage colony-stimulating factor (50 ng/mL) (Sigma), in 16-well slide-chambers (2×10^5 DC/well), as previously described [24]. hMΦ and DC phenotypes were routinely assessed by flow cytometry.

2.3. Normoxic and hypoxic conditions

Hypoxic cell culture conditions were established, as described previously [25]. The cell cultures were placed in a gas-tight modular chamber (Billups-Rothenberg, Del Mar, CA); the chamber was gassed for at least 15 min at a flow rate of 2 L/min using certified gases containing CO_2 and N_2 (White-Martins Gases, Rio de Janeiro, Brazil) and placed in a 37 °C temperature-controlled incubator. The percentage of O_2 was verified by measuring the outflow of gas at the end of the initial flushing period and then at 24 h intervals using a Fyrite apparatus (Bacharach, Inc., Pittsburgh, PA). The oxygen tension in the culture medium under hypoxic conditions was 7 mmHg and it was 150 mmHg under normoxic conditions (O_2 Analyzer YSI/53, Yellow Springs Instruments Inc., Yellow Springs, OH). In all experiments, cell exposure to <1% O_2, 5% CO_2, and balanced N_2 is referred to as hypoxia, and cell exposure to 21% O_2, 5% CO_2, and balanced N_2 is referred to as normoxia. The medium pH was 7.4 and did not change significantly during the course of the experiments.

2.4. Macrophage infection

Mouse MΦ were infected with *L. amazonensis* amastigotes (3:1 parasites/host cell) for 1 h, as previously described [26]. After the interaction period, the cultures were washed to remove extracellular parasites and fresh medium was added to the cell culture. Human MΦ and DC were infected with *L. amazonensis* amastigotes (3:1 parasites/host cell) for 24 h, as previously described [24]. Mouse and human MΦ and DC were infected with promastigote forms (10:1 parasites/host cell) for 24 h. Infected cell cultures were incubated in either normoxic or hypoxic conditions at 37 °C.

2.5. Cadmium chloride ($CdCl_2$) assay

In the HIF-1α inhibition experiments, $CdCl_2$ [27,28] were added to the mMΦ cultures that were infected in normoxic conditions for 24 h. Alternatively, $CdCl_2$ were added to the mMΦ cultures 1 h before the period of interaction with *L. amazonensis*. After the period of infection, the cells were stained with Giemsa and microscopically examined at 1000× magnification to evaluate the percentage of infected MΦ and the number of parasites per MΦ [21]. $CdCl_2$ treatment caused no toxicity to the infected cells at a concentration of 25 μM, as the treated cells presented the same morphology as nontreated cells.

2.6. HIF-1α immunofluorescence

Cells attached to the slide-chambers were fixed for 10 min with 4% paraformaldehyde and washed 3× in PBS. The cells were permeabilized with 1% Tween 20 and then washed 2× in PBS. Nonspecific binding sites were blocked with 3% BSA (Amresco, Solon, OH) for 30 min. The cells were then incubated with mouse anti-HIF-1α antibody diluted 1:100 (Abcam Inc., Cambridge, MA) (hMΦ and DC) or rabbit anti-HIF-1α antibody diluted 1:80 (Santa Cruz Biotechnology) (mMΦ) overnight at 4 °C in a wet room. The cells were washed 4× in PBS + 0.1% Tween 20 and incubated with FITC-conjugated goat anti-mouse secondary antibody diluted 1:100 (Sigma) or FITC-conjugated goat anti-rabbit secondary antibody diluted 1:60 (Sigma) for 1 h in a wet room at room temperature. The cells were washed 4× in PBS + 0.1% Tween 20 and mounted with DAPI-containing DABCO mounting media (Sigma). The cells were visualized under a Nikon Eclipse 50*i* fluorescence microscope (Nikon Inc., Melville, NY). All images were captured and analyzed with a digital camera (Nikon DXM1200-F) and imaging software (ACT-1, Nikon).

2.7. Detection of cellular hypoxia

Cellular hypoxia was detected by adding 200 μM of pimonidazole hydrochloride (Hypoxyprobe-1, Chemicon) to the cell cultures for 24 h. The cells were fixed for 10 min with 4% paraformaldehyde and washed 3× in PBS. Nonspecific binding sites were blocked with 3% BSA + 0.2% Brij (Sigma) for 15 min. The cells were then incubated with mouse anti-pimonidazole antibody diluted 1:40 (Chemicon) for 1 h at room temperature in a wet room, washed 3× in PBS + 0.2% Brij and incubated with FITC-conjugated goat anti-mouse antibody diluted 1:100 (Sigma) for 1 h at room temperature in a wet room. The cells were washed 3× in PBS + 0.1% Brij and slide-chambers were mounted with DAPI-containing DABCO mounting media (Sigma). The cells were visualized as described in Section 2.6.

2.8. Results analyses

The immunofluorescence experiments (HIF-1α and pimonidazole) were repeated at least 10 times. The $CdCl_2$ experiments were repeated at least three times and the results expressed as mean ± standard deviation. Statistical analyses were performed using the Student's *t*-test, with a significance level set at $P < 0.05$.

3. Results

3.1. HIF-1α immunofluorescence analyses

Immunofluorescence studies were performed to localize HIF-1α protein expression in mononuclear phagocytes. As shown in Fig. 1, in normoxic cultures, very weak immunofluorescence was detected in the cytoplasm and nuclei of mMΦ and hMΦ. Interestingly hDC cultured under normoxia showed HIF-1α staining. As might be expected, upon hypoxic exposure, all three mononuclear phagocyte types responded with a strong increase in HIF-1α expression (Fig. 1). HIF-1α was expressed more intensely in the nucleus than in the cytoplasm of mMΦ, hMΦ and hDC.

Various studies have shown that HIF-1α is expressed in cultured cells after an exposure to a range of bacteria and the parasite *T. gondii* [14–19]. Thus, this study investigated whether *L. amazonensis* interfered with HIF-1α expression in vitro. The present experiments involved the incubation of parasites with mMΦ for 1 h and the examination of HIF-1α expression at 24 h post infection. Human MΦ and DC were infected with parasites for 24 h and immediately tested for HIF-1α expression. The two experimental conditions, one for mouse cells and the other for human cells, permitted efficient infection and intracellular establishment of the parasites, as attested by the high number of infected cells (about 80% for mMΦ and about 60% for human mononuclear phagocytes), the number of intracellular amastigotes (about 8 per mMΦ and 5 for human mononuclear phagocytes), and in previous studies by our group [21,26]. Accordingly, immunofluorescence analyses revealed the presence of HIF-1α in mononuclear phagocytes after *L. amazonensis* infection under normoxic conditions similar to the staining pattern of hypoxic noninfected cells (Fig. 1). The expression of HIF-1α in infected cells cultured in hypoxia was also apparent (Fig. 1). It should be noted that the expression of HIF-1α for infected hDC was more intense than HIF-1α expression for noninfected DC maintained in normoxia. The HIF-1α protein was mainly detected in the nucleus of *L. amazonensis*-infected mononuclear phagocytes. These cells showed some diffuse cytoplasmic fluorescence with no staining of amastigote harboring vacuoles, which were identified as clear areas of cytoplasm (Fig. 1). Since no labeling was associated with intracellular amastigotes, we suggest that the HIF-1α antibodies detected a host cell protein rather than a parasite antigen. Lack of parasite staining was confirmed in amastigotes isolated from mMΦ cultures or mouse cutaneous lesions. In these preparations amastigotes were HIF-1α negative (data not shown). Taken together these experiments indicated that *L. amazonensis* amastigote infection results in HIF-1α expression in the host cells. *Leishmania* exists in two forms, amastigote and promastigote, and the same HIF-1α immunostaining pattern in amastigote-infected cells (Fig. 1) was found in promastigote-infected cells (data not shown). These results indicate that mononuclear phagocyte HIF-1α expression occurs regardless of the parasite stage used for infection.

3.2. Immunofluorescence analyses with pimonidazole as a hypoxic marker

Since hypoxia is a potent activator and regulator of HIF-1α, the question of whether infection with *L. amazonensis* led to cellular hypoxia was evaluated, using pimonidazole as a surrogate marker of hypoxia [29]. This substance creates adducts with thiol-containing proteins in hypoxic cells [16,30–32]. When FITC-labeled antibodies are bound to these complexes produced in hypoxia, fluorescence microscopy can be used to detect hypoxic cells [31]. As shown in Fig. 2, pimonidazole staining was virtually absent in mMΦ, hMΦ, and hDC under normoxic conditions. When the cells were exposed to hypoxia, pimonidazole staining was easily detectable (Fig. 2). Furthermore, intense cytoplasmic immunostaining was also observed in *L. amazonensis*-infected cells cultured under hypoxic conditions (Fig. 2). In contrast, pimonidazole staining was negative within infected cells maintained in normoxic conditions (Fig. 2). These results demonstrate that infection with *L. amazonensis* did not lead to a substantial decrease in oxygen concentration.

3.3. Effect of $CdCl_2$ on *L. amazonensis*-infected MΦ

To address the question of whether the inhibition of HIF-1α affected MΦ susceptibility to *L. amazonensis*, amastigotes were added to mMΦ for 1 h in normoxic conditions to permit parasite invasion. Next, the cell cultures were washed to remove extracellular parasites and treated with 25 μM $CdCl_2$, an inhibitor of HIF-1α [27,28]. As shown in Fig. 3 mMΦ were efficiently infected with *L. amazonensis* amastigotes (around 80% of infected cells and seven intracellular parasites per infected cell). Mouse MΦ infected with the parasite and treated with $CdCl_2$ showed about 40% reduction in both the percentage of infection (Fig. 3A) and the number of intracellular parasites (Fig. 3B) compared with nontreated cells. To test whether HIF-1α is required for parasite invasion, the mMΦ cultures were treated with $CdCl_2$ 1 h before the period of interaction with *L. amazonensis* and, after the period of interaction, infected cultures were incubated with $CdCl_2$ during 24 h in normoxia. Similarly, $CdCl_2$ treatment of mMΦ before *L. amazonensis* infection reduced the percentage of infected cells (Fig. 3A) and the number of intracellular parasites (Fig. 3B). Interestingly, 50 μM $CdCl_2$ was toxic to *L. amazonensis*-infected mMΦ but not to uninfected macrophages as observed by microscopy inspection (data not shown). These data indicate that HIF-1α activation is, at least partially, important for mMΦ support of *L. amazonensis* development and survival of MΦ.

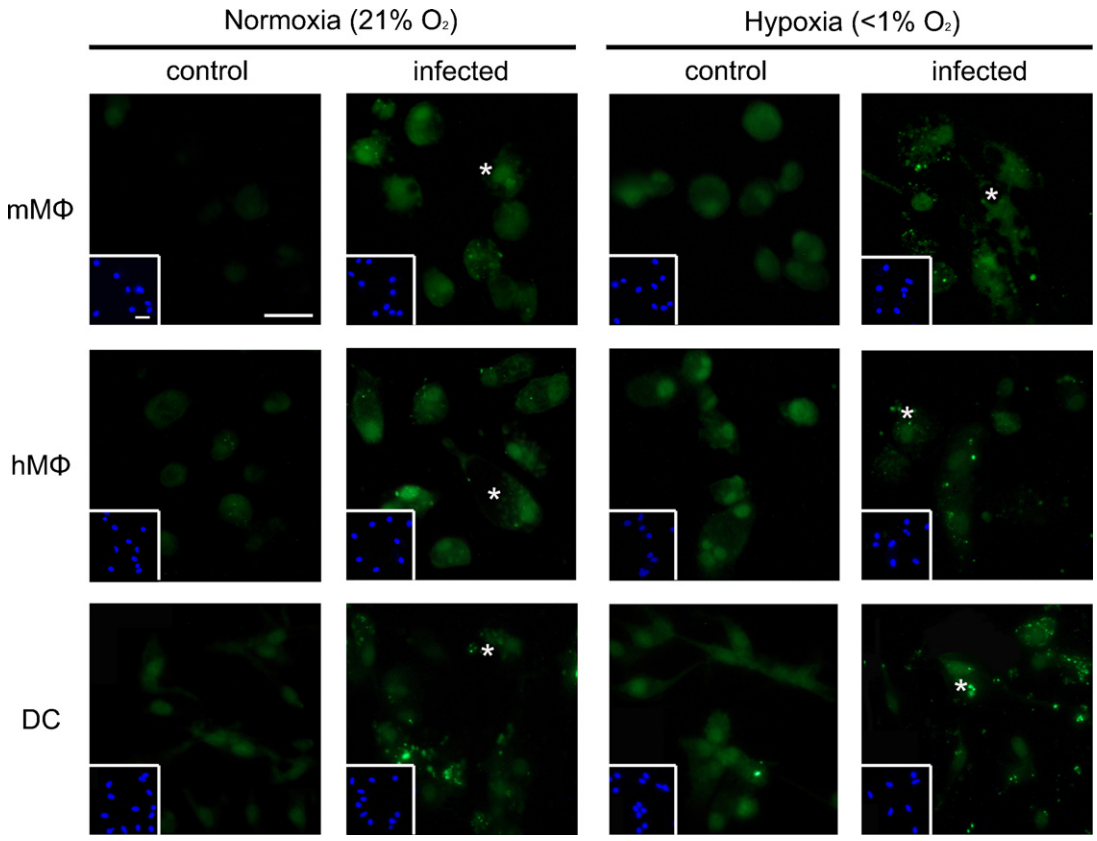

Fig. 1. Expression of HIF-1α by mouse macrophages (mMΦ) and human macrophages (hMΦ) and human dendritic cells (DC). The cells were infected with *L. amazonensis* amastigotes or left uninfected (control) and exposed to normoxia or hypoxia for 24 h, as described in Section 2. The cells were fixed and stained with anti-HIF-1α antibodies. Isotype and secondary antibody controls were negative for staining (data not shown). Insets show cell nuclei stained with DAPI (bars = 50 μm). *L. amazonensis*-harboring parasitophorous vacuoles are marked by asterisks.

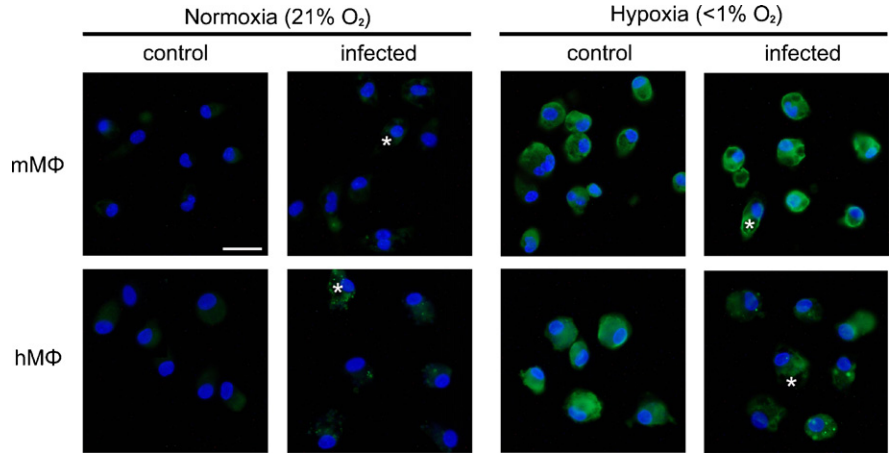

Fig. 2. Pimonidazole staining of mouse macrophages (mMΦ) and human macrophages (hMΦ). Cells were infected with *L. amazonensis* amastigotes or left uninfected (control), treated with pimonidazole (200 μM) and exposed to normoxia or hypoxia for 24 h as described in Section 2. The cells were fixed and stained with anti-pimonidazole complex antibody. Isotype and secondary antibody controls were negative for staining (data not shown). Insets show cell nuclei stained with DAPI (bar = 50 μm). *L. amazonensis*-harboring parasitophorous vacuoles are marked by asterisks.

4. Discussion

Despite the fact that hypoxia is the main activating factor for HIF-1α, an increasing body of evidence indicates that HIF-1α can be upregulated in different cell types by nonhypoxic stimuli, such as growth factors (insulin growth factor-1), cytokines (tumor necrosis factor (TNF-α), interleukin 1β (IL1β)), nitric oxide and lipopolysaccharides [11,13,14,33–37]. Pathogens such as *B. henselae*, *Streptococcus*, *S. aureus*, *Pseudomonas*, *Salmonella*, *C. pneumoniae*, Epstein Barr virus and the eukaryotic parasite *T. gondii*, lead to HIF-1α expression in different cell types under in vitro normoxic conditions [14–19]. Recently,

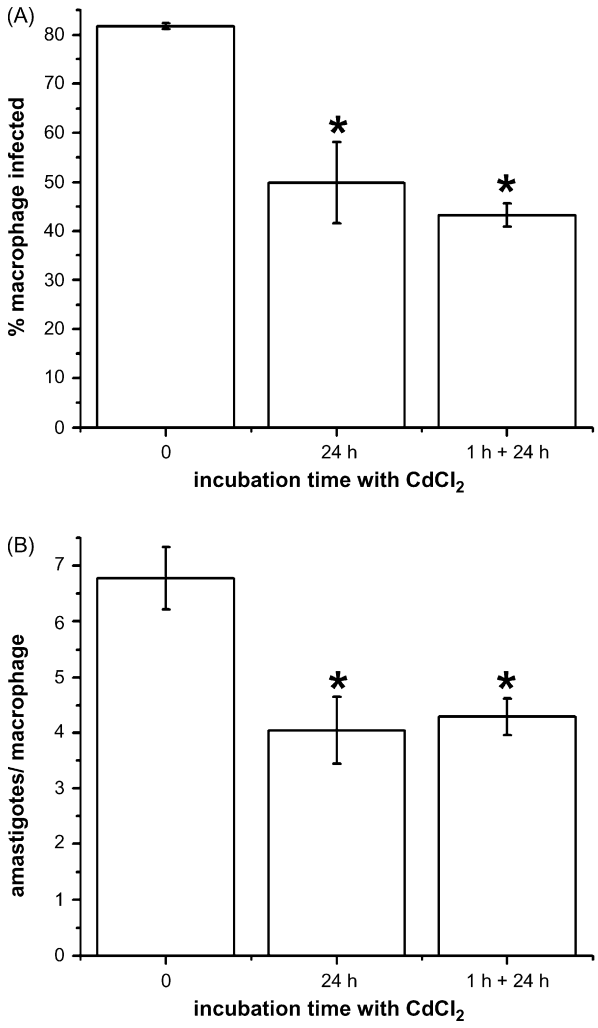

Fig. 3. Effect of $CdCl_2$ on *L. amazonensis*-infected MΦ. Mouse MΦ were infected with *L. amazonensis* amastigotes for 1 h and then washed to remove extracellular parasites. Twenty-five micromolars of $CdCl_2$ were added to the cell cultures, which were incubated in normoxia at 37 °C for 24 h (24 h). Alternatively, cell cultures were treated with $CdCl_2$ 1 h before the period of interaction with *L. amazonensis*, and then incubated in normoxia at 37 °C for 24 h (1 h + 24 h). The percentage of infected macrophages (A) and the number of amastigotes per macrophage (B) were determined as described in Section 2. The results represent the mean ± S.D. of one representative experiment out of three independently performed experiments with similar results. The significance of the differences between cell cultures treated or not with $CdCl_2$ is indicated in the figure: $^{*}P < 0.05$.

our group showed that HIF-1α is expressed in mouse cutaneous lesions during infection with *L. amazonensis*, an exclusive parasite of mononuclear phagocytes [5]. In this report, the expression of HIF-1α and a chemical marker of hypoxia (pimonidazole) in primary cultures of MΦ and DC infected with *L. amazonensis*, were analyzed. These in vitro cell systems are very useful for studying the cell biology of the host–parasite interactions [38,39]. In the experiments reported here, mMΦ, hMΦ and hDC were easily infected with *L. amazonensis* amastigote or promastigote forms and supported the infection without rapid cell degeneration. Large parasitophorous vacuoles, typically produced by this *Leishmania* species in MΦ and DC, facilitated microscopic analyses by visualization and counting [39,40].

Before elaborating on HIF-1α expression in *L. amazonensis*-infected mononuclear phagocytes, it should be noted that, to the best of our knowledge, these are the first experiments concerning the examination of HIF-1α expression in DC. It was of some interest to observe that HIF-1α was detected in the nucleus of noninfected DC under normoxic conditions, whereas HIF-1α was not detected in noninfected mMΦ and hMΦ when cultured in normoxia. This parallels the findings that HIF-1α is present at detectable levels under normoxia in some skeletal cells and during PMA-mediated differentiation in monocytic cell lines (THP1 and U937) [41–43]. In addition, cytokines, such as TNF-α, interferon-γ, IL1β and IL4 increase HIF-1α mRNA and/or protein levels during normoxia [44]. Ascertaining whether HIF-1α expression in DC under normoxic conditions is associated with the presence of IL4 and GM-CSF in the culture medium during the induction of DC differentiation should be the object of further investigation.

Only one other study has reported HIF-1α activation by protozoan infection. Spear et al. [18] showed HIF-1α accumulation in the cytoplasm and nuclei and the activation of HIF-1α reporter gene expression in human fibroblasts, Hela cells and RAW264.7 MΦ after *T. gondii* infection. The present experimental data revealed that HIF-1α immunoreactivity is primarily elevated in nuclei of mMΦ infected with *L. amazonensis*. Similar patterns of staining were obtained with *L. amazonensis*-infected hMΦ and DC. The most logical explanation for HIF-1α activation during infection is that parasite oxygen consumption leads to a localized hypoxic response in the host cell. Although cellular hypoxia was not determined in *T. gondii*-infected cells [18], a recent study showed that HIF-1α activation by *B. henselae*-infected cells was accompanied by cellular hypoxia, as revealed by pimonidazole staining [16]. Present data indicated that pimonidazole adducts were barely detectable in *L. amazonensis*-infected mononuclear phagocytes under normoxic conditions. These results suggest that substantial hypoxia does not develop during *L. amazonensis* infection in vitro. Since pimonidazole adducts are formed when O_2 tension is below 1–2% O_2, with half-maximal binding around 0.1% O_2 [45–47], and HIF-1α half-maximal activation in cultured cells requires O_2 tension between 1.5 and 2% O_2, with maximal response at 0.5% O_2 [48], the current results suggest that HIF-1α expression in *L. amazonensis*-infected mononuclear phagocytes may be mostly independent of hypoxia. Consequently, nonhypoxic stimuli must be involved in *L. amazonensis* activation of HIF-1α. Spear et al. [18] proposed that a short lived diffusible factor signal to the host cell, such as reactive oxygen species (ROS), activated HIF-1α in *T. gondii* infection, based on studies showing that nitric oxide and superoxide are induced by infection [49] and the fact that studies have demonstrated that ROS activates HIF-1α [50,51]. This appears to differ from *Leishmania*, which reportedly suppresses the oxidative burst and nitric oxide production during in vitro infection [52,53]. The possibility that proinflammatory cytokines, such as TNF-α, involved in wound MΦ and neutrophil HIF-1α induction [11], are also involved in *L. amazonensis*-infected cell HIF-1α induction is unlikely because *Leishmania* downregulates the expression of proinflammatory cytokines during infection (Degrossoli and Giorgio, unpublished data; Refs. [53–55]).

Despite the uncertainties regarding how HIF-1α is activated during *L. amazonensis* infection, the picture emerging from these studies is that HIF-1α induction and the target genes likely constitute part of an adaptative mechanism resulting from parasite infection and that this could permit the host cell to attenuate damage, maintain integrity and survive such infection. This conclusion is supported by data reported previously obtained in *T. gondii*-infected HIF-1α knockout cells where parasite survival was partially reduced [18], and by the results shown here, indicating that the addition of a HIF-1α inhibitor, $CdCl_2$ [27,28], to *L. amazonensis*-infected MΦ cultures partially reduced parasite survival. It is also noteworthy that *L. amazonensis* infection of MΦ rendered them more susceptible to high dose $CdCl_2$ treatment. Full understanding of HIF-1α involvement in infectious diseases should contribute to elucidating the adaptative and immune responses of the host to parasitic invasion.

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