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The Influence of Low Oxygen on Macrophage Response to *Leishmania* Infection

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Abstract

Hypoxia (low oxygen tension) is a common feature of inflamed and infected tissues. The influence of hypoxia on macrophage responses to micro-organisms has only recently been studied. This study demonstrates that hypoxia induced macrophages to control *Leishmania amazonensis,* an intracellular parasite that causes cutaneous and cutaneous metastatic lesions. The mechanisms that contribute to the control of macrophages against *L. amazonensis* infection under a hypoxic microenvironment are not known. Nitric oxide, TNF-α, IL-10 or IL-12 is not responsible for the decrease in parasitism under hypoxia. Live *L. amazonensis* entry or exocytosis of internalized particles as well as energetic metabolism was not impaired in infected macrophages; no apoptosis-like death was detected in intracellular parasites. Reactive oxygen species (ROS) is likely to be involved, because treatment with antioxidants *N*-acetylcysteine (NAC) and ebselen inhibits the leishmanicidal effect of macrophages under hypoxia. *Leishmania amazonensis* infection induces macrophages to express hypoxia-inducible factor-1 (HIF-1α) and -2 (HIF-2α). Data indicate that hypoxia affects the microbial activities and protein expression of macrophages leading to a different phenotype from that of the normoxic counterpart and that it plays a role in modulating *Leishmania* infection.

Introduction

Hypoxia (low oxygen tension) is a common feature in tumours, wounds, atherosclerotic lesions and inflamed or infected tissues [1, 2]. Several characteristics shared by diseased tissues, such as increased metabolic demand for leucocytes infiltrated into the inflammatory tissue, microcirculation impairment and cell and/or micro-organism proliferation, result in a hypoxic environment [3]. Macrophages, cells involved in the clearance of micro-organisms, antigen processing/presentation and angiogenesis, adapt to hypoxia by modulating their cytokine secretion, expression of cell surface markers, migration, pinocytosis and phagocytosis [3, 4]. The influence of low oxygen tension on macrophage responses to micro-organisms has only recently been studied [5, 6]. In previous studies [7–11], our group has demonstrated that hypoxia induced macrophages to control *Leishmania amazonensis*, an intracellular parasite that causes cutaneous and cutaneous metastatic lesions [12, 13], and express hypoxia-inducible factor-1 (HIF-1α), a transcriptional protein controlled mainly by hypoxia [10, 11]. Besides leishmaniasis, other protozoan, bacterial and viral infections can be modulated by hypoxia and activate the HIF system [5, 6]. The mechanisms by which macrophages are able to control *L. amazonensis* infection during hypoxia are unknown. In this study, several cellular processes were investigated, including nitric oxide and ROS involvement, cytokine production, phagocytosis, exocytosis, ATP release, HIF expression and whether apoptosis occurred in intracellular parasites inside macrophages under hypoxia.

Materials and methods

Animals. Female BALB/c, C57Bl/6 and C57Bl/6 iNOS knockout (deficient in iNOS) mice, aged 8–12 weeks, were obtained from Animal Center of the Universidade Estadual de Campinas, Campinas, SP, Brazil.

Cell cultures and parasites. The murine macrophage cell line J774 was maintained in RPMI 1640 medium supplemented with 25 μg/ml gentamicin, 2 mM L-glutamine, 10 mM HEPES (Sigma Aldrich, St Louis, MO, USA) and 10% foetal calf serum (Nutricel, Campinas, Brazil) at 37 °C in 5% CO_2, 5% O_2 and balanced N_2 [8, 9]. Primary mouse macrophages were obtained from normal BALB/c mice by peritoneal lavage, as described [8,

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9]. *Leishmania amazonensis* (MHOM/BR/73/M2269) amastigotes were isolated from active skin lesions of BALB/c mice, as described [14]. The parasites were suspended in RPMI 1640 medium and used immediately after isolation. The experimental protocols were approved by the Institute of Biology/Universidade Estadual de Campinas Ethical Committee for Animal Research.

Normoxic and hypoxic conditions. Hypoxic cell culture conditions were established as described [7, 8]. The cell cultures were placed in a gas-tight modular chamber (Billups-Rothenberg, Del Mar, CA, USA); the chamber was gassed for at least 15 min at a flow rate of 2 l/min, using certified gases containing O_2, CO_2 and N_2 (White-Martins Gases, Rio de Janeiro, RJ, 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, USA). The oxygen tension in the culture medium under hypoxic conditions was 7 or 37 and 150 mmHg under normoxic conditions (O_2 Analyzer YSI/53; Yellow Springs Instruments Inc., Yellow Springs, OH, USA). In all experiments, cell exposure to 1% or 6% 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 pH of all cell culture media was 7.4 and did not change significantly during the course of the experiments [7]. Cytotoxicity was analysed by the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye reduction assay [15] (Sigma).

Macrophage infection and assessment of intracellular parasites. Peritoneal macrophages and J774 cells were infected with *L. amazonensis* amastigotes at parasite–cell ratio of 3:1 and 10:1, respectively, for 1 h, as described [7, 9]. Briefly, following a period of interaction, the cultures were washed to remove extracellular parasites, and fresh medium was added to the cell cultures. To determine the percentage of infected macrophages and number of amastigotes per macrophage, cells on coverslips were stained with Giemsa and examined microscopically at 1000× magnification. About 600 cells were counted per triplicate coverslip [7].

Antioxidants. N-acetylcysteine (NAC) (Sigma), a thiol compound that acts as a direct ROS scavenger, was diluted in deionized sterile water and added to the cell cultures at a final concentration of 5 mM for 2 h at 37 °C before treatments [16, 17]. 2-Phenyl-1,2-benzisoselenazol-3(2H)-one (ebselen) (Calbiochem, La Jolla, CA, USA) that mimics glutathione peroxidase, a H_2O_2-detoxifying enzyme, was diluted in DMSO, and 40 μM (0.02% DMSO) was added to the cultures at the beginning of the experiments [18, 19].

Cytokine assays. The concentrations of IL-6, IL-10 and IL-12 in macrophage supernatants were determined by an enzyme-linked immunosorbent assay (ELISA) kit (Biosource, Camarillo, CA, USA), used in accordance with the manufacturer's instructions. The supernatants were assayed for TNF-α in a cytotoxicity assay using L929 cells pretreated with 5 μg/ml actinomycin D in 96-well microtiter plates, as previously described [9]. The cytotoxicity effects of supernatants containing TNF-α activity were calculated using the following formula: Cytoxicity (%) = $[OD_{540}\text{control} - OD_{540}\text{test}]/OD_{540}\text{control} \times 100$, where *control* and *test* represent the absorption of L929 plus medium and L929 plus supernatants, respectively. One unit of TNF activity equals 50% L929 cytotoxicity.

Ultrastructural analyses. For ultrastructural analyses, fixative consisting of 2.5% glutaraldehyde (Electron Microscope Science, Hatfield, PA, USA) in 0.1 M sodium cacodylate (Electron Microscope Science) buffer at pH 7.4 was added to the medium of adherent cells for 15 min. Then, the fixative and medium mixture was replaced by pure fixative for 45 min. Next, the cells were rinsed (3 × 10 min) in 0.1 M sodium cacodylate buffer, pH 7.4, and then post-fixed in 1% OsO_4 (Electron Microscope Science) solution for 1 h. Following dehydration in an ethanol gradient, adherent cells were embedded in Epon 812 resin (Electron Microscope Science). Ultra-thin sections were stained with uranyl acetate and lead citrate and observed in a LEO 906 (Leica, Oberkochen, Germany) transmission electron microscope operated at 60 kV.

Phagocytosis and exocytosis assays and the preparation of IgG-opsonized microspheres. Leishmania amazonensis phagocytosis was assayed with fresh or fixed amastigotes isolated from footpad lesions of BALB/c mice. The parasites were fixed for 60 min at 4 °C with 0.5% glutaraldehyde (Merck, Darmstadt, Germany) in PBS solution at 10^8 parasites/ml, washed three times with PBS, resuspended in PBS at 10^9 parasites/ml and maintained at 4 °C until use [20]. Peritoneal macrophages plated on 13-mm glass coverslips (5×10^5 cells/well) were incubated overnight at 37 °C and then cultured with living amastigotes (3:1 parasites/host cell) or fixed amastigotes (10:1 parasites/host cell). Following 1 h of incubation in normoxia or hypoxia, the cells were washed to remove extracellular parasites, and the coverslips were stained with Giemsa. Phagocytosis was quantified by morphological examination, evaluating the percentage of infected macrophages and the number of amastigotes per macrophage microscopically at 1000× magnification. About 600 cells were counted per triplicate coverslips [7].

In exocytosis assays, macrophages were exposed to 100 μl FITC-labelled *Escherichia coli* K-12 bioparticles® (1 mg/ml) (Molecular Probes, Invitrogen, Eugene, OR, USA) for 1 h under normoxia. Next, the extracellular bioparticles were removed, and fluorescence was measured. Macrophages were further incubated in normoxia or hypoxia, and fluorescence was measured after 1 and 24 h. The relative fluorescence was calculated using the following formula: $Ft/Fc \times 100$, where *Ft* is the fluores-

cence of the test sample and *Fc* is the fluorescence of macrophages in normoxia (control) at initial time.

TUNEL assay. *In situ* detection of DNA fragments by terminal deoxyribonucleotidyltransferase (TdT)-mediated dUTP nick ending labelling (TUNEL) was performed using Fluorescein FragEL™ DNA Fragmentation Detection kit (Calbiochem), in accordance with the manufacturer's instructions. Infected macrophages on coverslips were fixed for 10 min with 4% paraformaldehyde and washed three times in PBS, and then, the TUNEL assay was developed. The cells were counterstained with DAPI and visualized under a Nikon Eclipse 50*i* fluorescence microscope (Nikon Inc., Melville, NY, USA). All images were captured and analysed with a digital camera (Nikon DXM1200-F) and imaging software (ACT-1; Nikon). The percentage of labelled intracellular amastigotes was analysed by digitized images from microscopic fields.

ATP assay. Cellular ATP content was determined using a luciferase-based ATP assay kit ATPlite (Perkin-Elmer, Life and Analytical Sciences, Downers Grove, IL, USA), in accordance with the recommended procedure. Briefly, 100 μl of cell homogenate was added to a 96-well plate followed by 50 μl substrate buffer. Luminescence was counted using a luminescence spectrometer microplate reader (Synergy HT; Biotek, Winooski, VT, USA) after mixing and 10-min dark adaptation of the plate. ATP standards and blanks were incorporated in each reading.

HIF-1α and HIF-2α immunofluorescence. Cells attached to the slide chambers were fixed for 10 min with 4% paraformaldehyde and washed three times in PBS. The cells were permeabilized with 1% Tween 20 and then washed twice in PBS. Non-specific binding sites were blocked with 3% BSA (Amresco, Solon, OH, USA) for 30 min. The cells were then incubated with rabbit anti-HIF-1α and anti-HIF-2α antibodies (Abcam Inc., Cambridge, MA, USA and Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C in a wet room. The cells were washed three times in PBS + 0.1% Tween 20 and incubated with FITC-conjugated goat anti-rabbit secondary antibody diluted 1:100 (Sigma) for 1 h in a wet room at room temperature, washed three times 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.). All images were captured and analysed with a digital camera (Nikon DXM1200-F) and imaging software (ACT-1; Nikon).

Statistical evaluation. All experiments, except for immunofluorescence analyses, were repeated at least three times, and the results are expressed as the mean ± SD. The immunofluorescence experiments were repeated at least 10 times. Data obtained under different conditions (normoxia, hypoxia, non-infection and infection) were analysed statistically by the Student *t*-test, with the level of significance set at $P \leq 0.05$ for *in vitro* assays.

Results

Leishmania amazonensis infection

Macrophages were efficiently infected with *L. amazonensis* in normoxia (Fig. 1A, B). However, in macrophages subjected to hypoxia, a significant reduction occurred in the percentage of infected macrophages (decrease by 40–60%) and number of parasites inside the cells (decrease by 20–35%) compared with the normoxic condition (Fig. 1A, B). Viability was determined in cells exposed to normoxia and hypoxia; there was no significant dif-

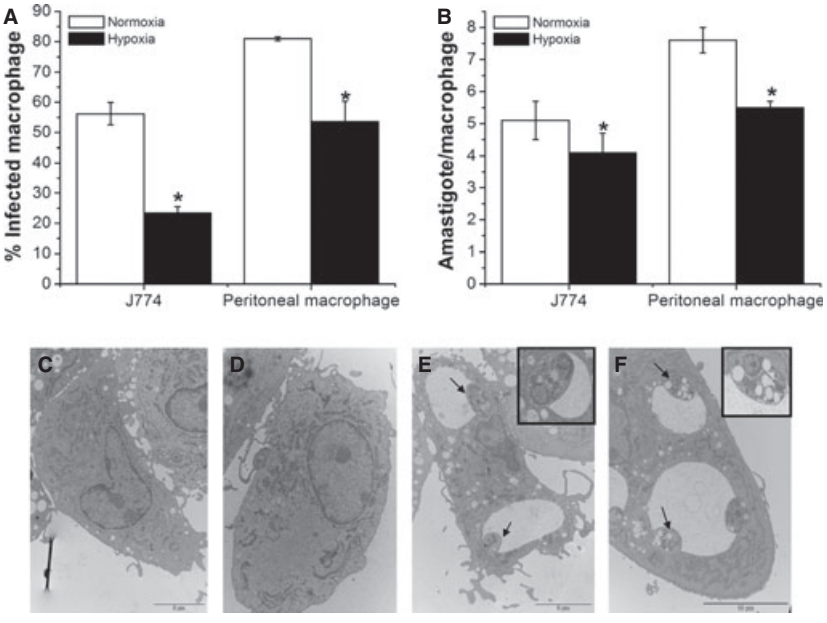

Figure 1 Effect of hypoxia on macrophages. Peritoneal or J774 macrophages were infected with *Leishmania amazonensis* amastigotes for 1 h, extracellular parasites were removed and cells maintained in normoxia or hypoxia for 24 h. The percentage of infected macrophages (A) and the number of amastigotes per macrophage (B) were determined. Ultrastructural appearance of uninfected macrophages in normoxia (C) and hypoxia (D) and *L. amazonensis*-infected macrophage under normoxia (E); arrow heads indicate amastigotes inside parasitophorous vacuoles. Inset shows a typical amastigote. *Leishmania amazonensis*-infected macrophage in hypoxia (F); arrow heads indicate amastigotes inside parasitophorous vacuoles. Inset shows amastigote presenting lipid inclusions and cytoplasmic vacuolization.

ference in the OD values obtained by the MTT assay (0.47 ± 0.09 of the normoxic infected J774 macrophages versus 0.37 ± 0.03 of the hypoxic infected J774 macrophages). There was also no significant difference in the viability between extracellular amastigotes preincubated under normoxia or hypoxia (OD values: 0.05 ± 0.009 versus 0.09 ± 0.03 and cell counts: $2 \times 10^6 \pm 1 \times 10^5$ versus $2.1 \times 10^6 \pm 3 \times 10^5$, respectively). Morphological appearance did not differ between uninfected cells under normoxia and hypoxia (Fig. 1C, D). The ultrastructure of infected macrophages under normoxia showed parasitophorous vacuoles containing amastigotes attached to the inner surface (Fig. 1E). The cells under hypoxia had vacuoles with few parasites or cells had vacuoles with parasites presenting lipid inclusions and cytoplasmic vacuolization (Fig. 1F), similar to those previously reported in squalene synthase inhibitors–treated *L. amazonensis* [21] and taxol-treated *Trypanosoma cruzi* [22], indicating that these ultrastructural changes can occur as a consequence of different perturbations to the parasite's cellular functions.

Nitric oxide involvement

Experiments that helped to evaluate whether nitric oxide is involved in the antileishmanial activity of macrophages under hypoxia were conducted with cells from iNOS knockout mice. Both cell systems i.e., macrophages from iNOS knockout and wild-type mice, were efficiently infected with *L. amazonensis* under normoxia (Fig. 2). The iNOS knockout and wild-type macrophages subjected to hypoxia showed a significant reduction in the percentage of infected macrophages and number of parasites inside the cells (Fig. 2). Previous results from our laboratory had shown that wild-type infected macrophages exposed to normoxia or hypoxia release low levels of nitrate [7]. These data suggest that nitric oxide is not involved in the antileishmanial activity of macrophages induced by hypoxia.

ROS involvement

Other molecules involved in macrophage killing of *Leishmania* spp, besides reactive nitrogen intermediates, are ROS [23–26]. Experiments tested the possibility of antioxidant compounds, such as NAC or ebselen, altering the infection level of macrophages in hypoxia. As expected, macrophages infected under hypoxia showed a reduction in the percentage of infected cells and number of amastigotes per cell compared with normoxic conditions (Fig. 3). Interestingly, the reduction in parasite level observed in macrophages under hypoxia was not observed in cells cultured with NAC under hypoxia. Similar results were observed with ebselen that is no reduction occurred in the parasite level in cells treated with the antioxidant under hypoxia (Fig. 3). These data suggest the participation of ROS in the antileishmanial activity of macrophages is induced by hypoxia.

Cytokine production

Cytokines are key elements in the host response against *Leishmania* sp. [13] TNF-α, IL-6, IL-10 and IL-12 production were all evaluated (Fig. 4). Two classical agents that when added together induce proinflammatory cytokines TNF- and IL-12 and when added alone (LPS) induce IL-6 and IL-10 [27] were used as control of tests. TNF-α bioactivity was not enhanced by hypoxia in infected macrophages, although the cytokine bioactivity was enhanced in cells non-stimulated and stimulated with IFN-γ + LPS under hypoxia compared with cells cultured in the same conditions and exposed to normoxia (Fig. 4A). Interestingly, a modest but significant increase in IL-6 production was detected in infected macrophages under hypoxia compared with infected cells under normoxia (Fig. 4B); higher concentrations of IL-6 were detected in LPS-treated macrophages under hypoxia. Regarding IL-12, little or no production was observed in non-infected or infected macrophages cultured under normoxia or hypoxia; when

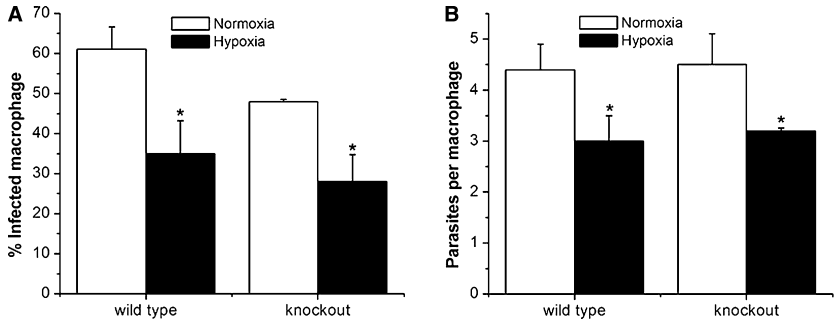

Figure 2 Effect of hypoxia on *Leishmania amazonensis*-infected macrophages. Peritoneal wild-type and iNOS knockout macrophages were infected with *L. amazonensis* amastigotes for 1 h, extracellular parasites were removed and cells maintained in normoxia or hypoxia for 24 h. The percentage of infected macrophages (A) and the number of amastigotes per macrophage (B) were determined. The result represents the mean ± SD of three experiments. The significance of the difference between cell cultures in normoxia and hypoxia is indicated: $*P \leq 0.05$.

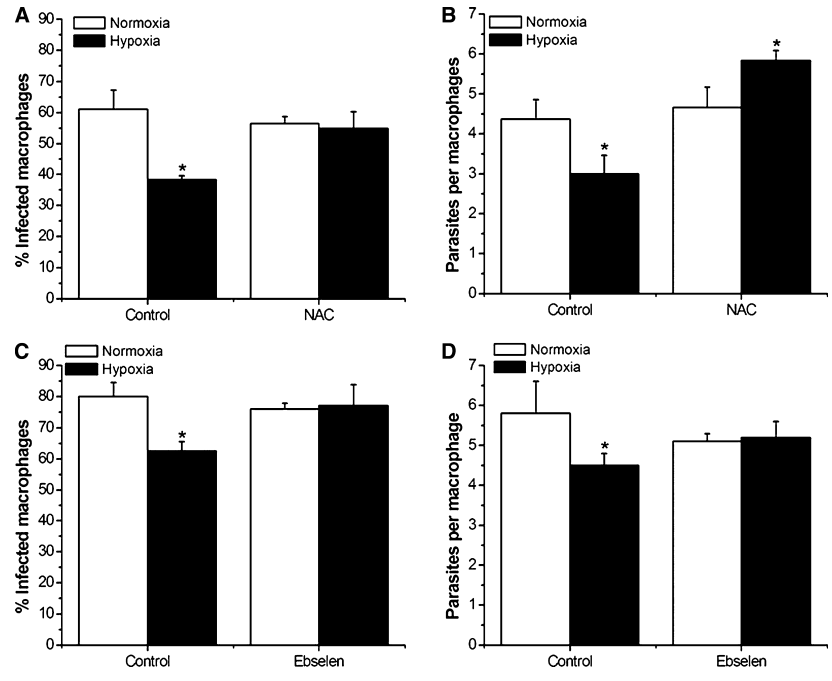

Figure 3 Effect of antioxidants on *Leishmania amazonensis*-infected macrophages. Peritoneal macrophages were infected with *L. amazonensis* amastigotes for 1 h in normoxia, extracellular parasites were removed and cells maintained in normoxia or hypoxia with antioxidants NAC or ebselen for 24 h. The percentage of infected macrophages (A and C) and the number of amastigotes per macrophage were determined (B and D). The result represents the mean ± SD of three experiments. The significance of the difference between cells cultures in normoxia and hypoxia is indicated: $*P \leq 0.05$.

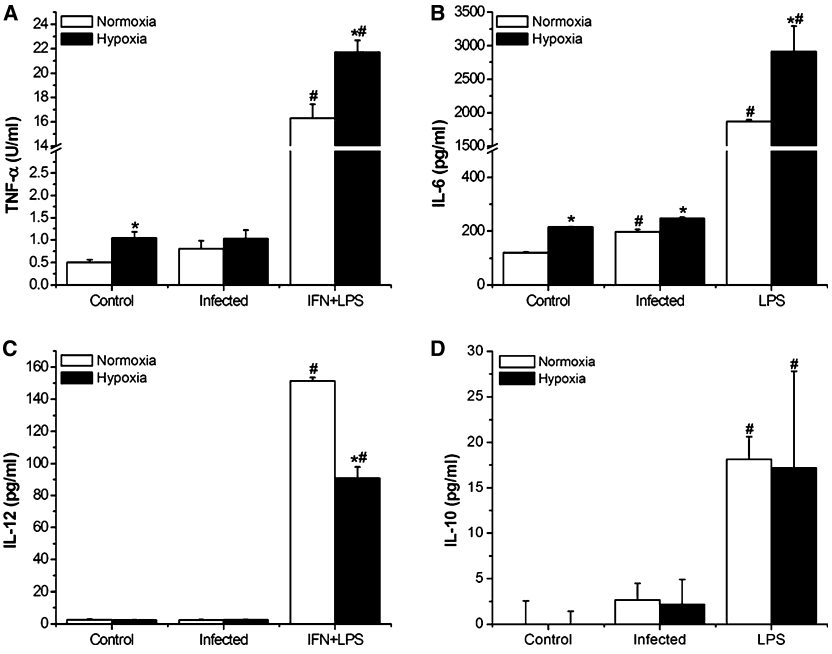

Figure 4 Effect of hypoxia on cytokine release by macrophages. Peritoneal macrophages were treated with LPS, IFN-γ + LPS or infected with *Leishmania amazonensis* amastigotes and incubated for 24 h under normoxia or hypoxia. TNF-α activity was determined in the culture supernatants by L929 cytotoxicity assay (A), and IL-6 (B), IL-12 (C) and IL-10 (D) production was determined in the supernatants by ELISA. The results represent the mean ± SD of three experiments. *The significance of the difference between cell cultures in normoxia and hypoxia. #The significance of difference between infected or stimulated cell cultures and control. $P \leq 0.05$.

macrophages were stimulated with IFN-γ + LPS in normoxia, they produced IL-12 at higher concentrations than cells stimulated with IFN-γ + LPS under hypoxia (Fig. 4C). A modest IL-10 production was detected in infected cells under normoxia or hypoxia, but high concentrations of this cytokine were detected in macrophages stimulated with LPS under normoxia or hypoxia (Fig. 4D).

Effects of hypoxia on phagocytosis and exocytosis

To explore the hypothesis that macrophages under hypoxia reduce *L. amazonensis* infection because microorganism uptake by cells is impaired within this microenvironment, the phagocytosis of living and dead parasites was analysed. To achieve this, the parasites were cultured with cells for 1 h to permit invasion. Although phagocytosis of dead parasites that is glutaraldehyde-fixed parasites by macrophages under hypoxia was reduced (Fig. 5A, B), living parasites were efficiently internalized by macrophages cultured under normoxia or hypoxia as demonstrated by the percentage of infected cells and the average number of intracellular amastigotes (Fig. 5A, B). These data excluded the possibility that an impaired parasite uptake is involved in the low levels of infection in

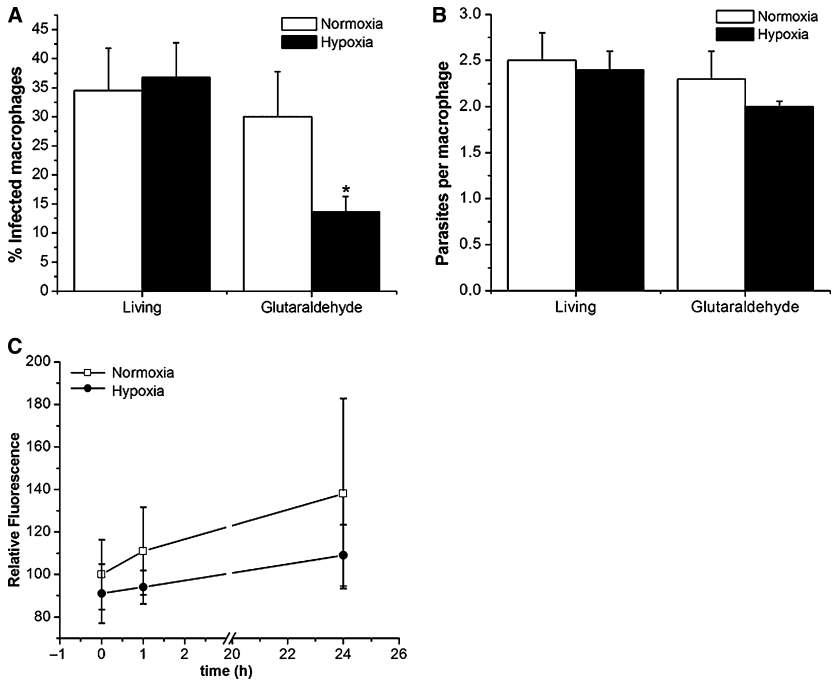

Figure 5 Effect of hypoxia on phagocytosis and exocytosis by macrophages. Peritoneal macrophages were incubated with living or glutaraldehyde-fixed *Leishmania amazonensis* amastigotes and cultured in normoxia or hypoxia for 1 h. Extracellular amastigotes were removed, and the percentage of infected macrophages (A) and the number of amastigotes per macrophage (B) were determined. Peritoneal macrophages were incubated with FITC-labelled *Escherichia coli* K-12 bioparticles for 1 h under normoxia (C). Cell cultures were washed to remove extracellular particles and were further incubated for 1 and 24 h in normoxia or hypoxia. Intracellular particles were quantified by measuring the fluorescence intensity of the cells. The fluorescence was normalized to the fluorescence of macrophages in normoxia at initial time (0 h). The result represents the mean ± SD of three experiments. The significance of the difference between cell cultures in normoxia and hypoxia is indicated: $*P \leq 0.05$.

macrophages under hypoxia. Another hypothesis to explain *L. amazonensis* reduction in macrophages under hypoxia is that parasites/particles could be released from cells under hypoxia, a process denominated exocytosis [28]. Experiments to test this hypothesis were conducted with macrophages incubated with FITC-labelled *E. coli* K-12 bioparticles under normoxia or hypoxia. The cells showed similar relative fluorescence after 1 and 24 h under normoxia and hypoxia, suggesting that internalized particles are maintained inside the cells and that exocytosis did not occur in hypoxic macrophages (Fig. 5C).

ATP production

An ATP bioluminescence assay was used to evaluate whether exposing macrophages to hypoxia affected total cellular ATP levels (Fig. 6). Incubation of uninfected or infected cells under normoxia or hypoxia (6% O_2) resulted in similar ATP levels (Fig. 6A); similarly, 1% O_2 causes no appreciable decrease in ATP levels in infected macrophages, but diminished ATP levels were observed in uninfected macrophages (Fig. 6B).

DNA fragmentation on intracellular amastigotes

As leishmanicidal activity in macrophages stimulated with IFN-γ + LPS is related to apoptosis-like death in parasites inside the macrophages [29], we investigated whether the same phenomena occurred in infected cells under hypoxia. The TUNEL assay was used to detect nuclear DNA fragmentation in cell cultures. As expected, apoptosis of intracellular amastigotes did not occur in

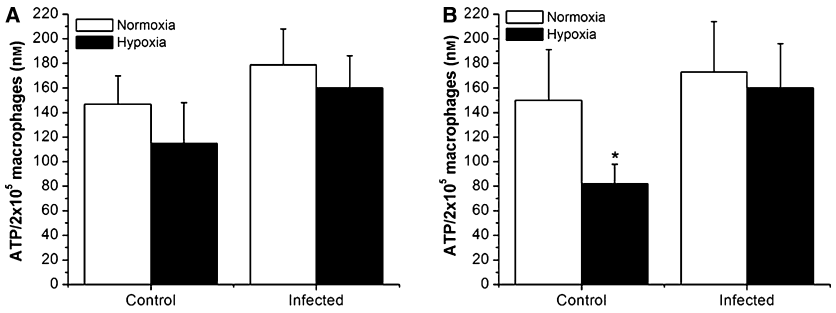

Figure 6 Effect of hypoxia on ATP release by macrophages infected with *Leishmania amazonensis*. Peritoneal macrophages were infected with *L. amazonensis* amastigotes and incubated for 24 h under normoxia or hypoxia with 6% O_2 (A) or 1% O_2 (B). ATP concentrations were determined in the supernatants by the bioluminescence assay. The results represent the mean ± SD of three experiments. The significance of the difference between cell cultures in hypoxia and normoxia is indicated: $*P \leq 0.05$.

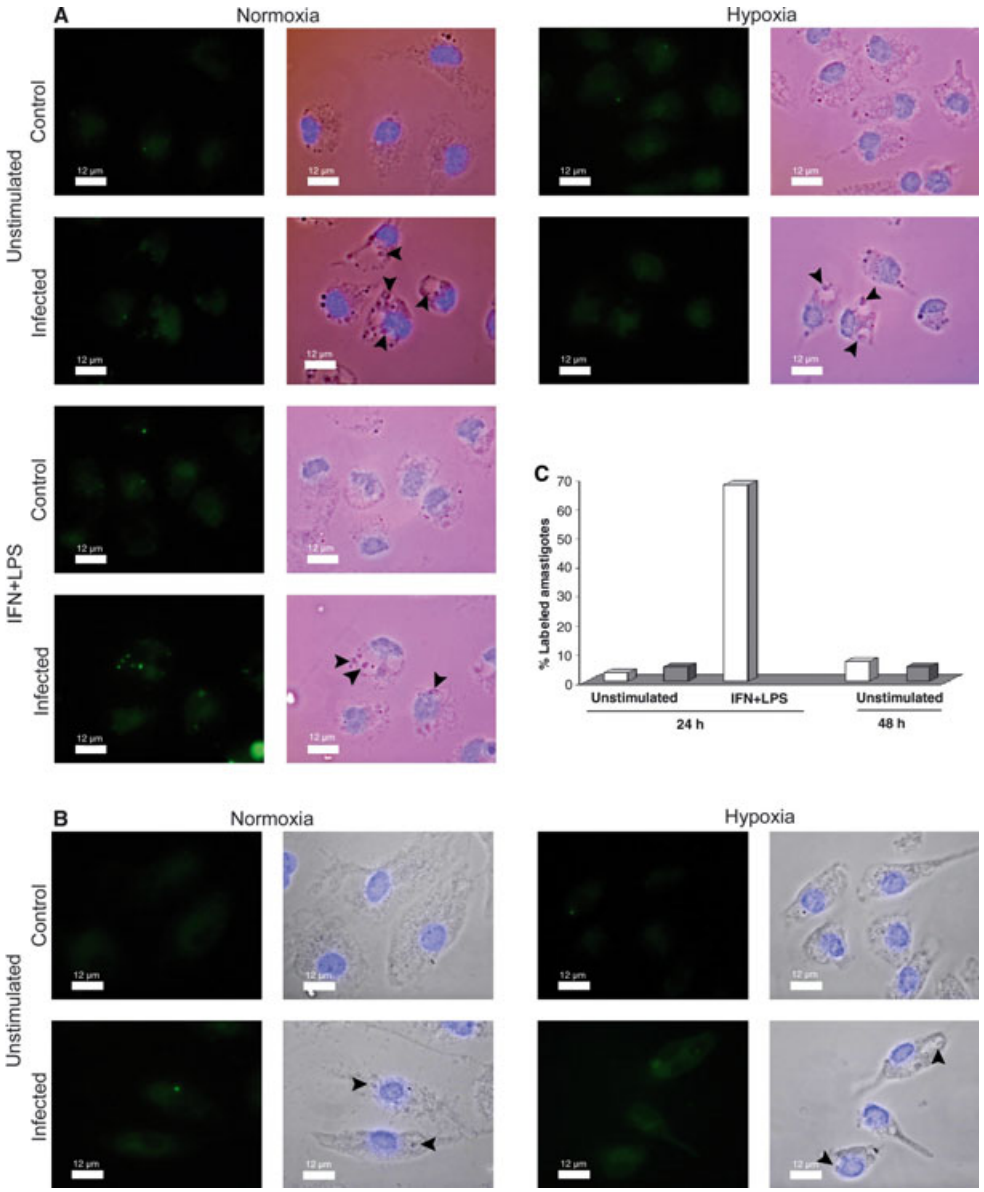

Figure 7 *In situ* analysis of apoptosis in *Leishmania amazonensis*-infected macrophages. Peritoneal macrophages were infected with *L. amazonensis* amastigotes and incubated under normoxia or hypoxia for 24 h (A), 48 h (B) or stimulated with IFN-γ + LPS in normoxia for 24 h (panel A). DNA fragmentation analysis was determined by the TUNEL method and analysed under a fluorescent and phase contrast microscope (bars = 12 μm). Cell nuclei were stained with DAPI and merged with phase contrast. *L. amazonensis*-harbouring parasitophorous vacuoles are indicated by arrowheads. Bars represent the percentage of labelled intracellular amastigotes with DNA fragmentation (C).

macrophages under normoxia, because the parasites are viable and multiply inside host cells (Fig. 7). Apoptosis also did not occur in parasites inside cells under hypoxia (Fig. 7A, C). In contrast, IFN-γ + LPS–induced DNA fragmentation was visualized in 67% of intracellular amastigotes (Fig. 7C).

HIF-1α and HIF-2α expression

Immunofluorescence studies were performed to locate the HIF system in macrophages. As expected, very weak HIF-1α and HIF-2α fluorescence was detected in the cytoplasm and nucleus of uninfected macrophages under normoxia (Fig. 8). Following hypoxic exposure, uninfected macrophages responded with a strong increase in HIF-1α and HIF-2α expression; and as previously reported by our group [10], HIF-1α was expressed in infected cells under normoxia or hypoxia (Fig. 8). Interestingly, immunofluorescence assays revealed the presence of HIF-2α in infected macrophages under normoxia and hypoxia. The HIF-2α was expressed more intensely in the nucleus than in the cytoplasm of macrophages (Fig. 8).

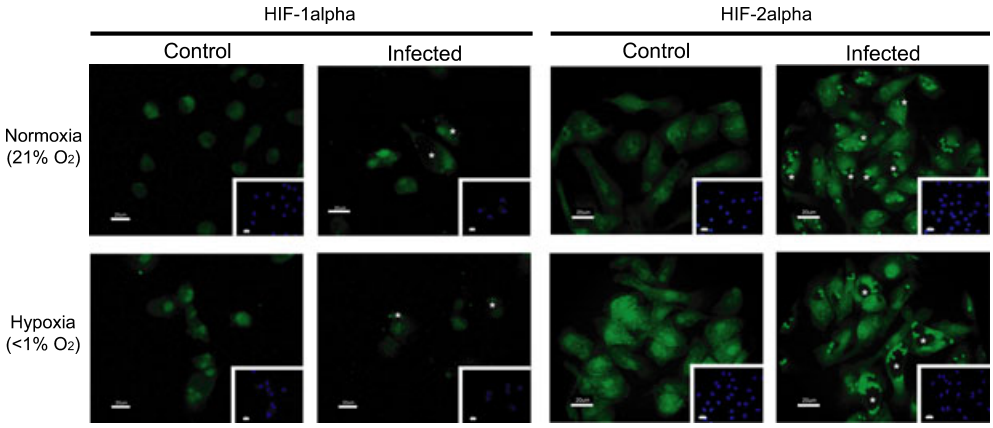

Figure 8 Expression of HIF-1α and HIF-2α by peritoneal macrophages. The cells were infected with *Leishmania amazonensis* amastigotes or left uninfected (Control) and exposed to normoxia or hypoxia for 24 h. The cells were fixed and stained with anti-HIF-1α or anti-HIF-2α antibodies. Isotype and secondary antibody controls were negative for staining (data not shown). Insets show cell nuclei stained with DAPI (bars = 20 μm). *L. amazonensis*-harbouring parasitophorous vacuoles are marked by asterisks.

Discussion

A number of recent studies have attempted to evaluate the impact of low oxygen on macrophages [4, 5]. Phenotypical and functional changes include the modulation of cytokines, such as IL-1, TNF-α and its receptors, IL-6, IL-18 binding protein, endothelial growth factor, iNOS, matrix metalloprotein-7 and NF-$\kappa\beta$ [4, 5, 30]. It has been accepted that macrophages adapt to hypoxia by redefining their transcriptome and acquiring a "hypoxic" phenotype substantially different from that of the normoxic counterpart [30]. In fact, at inflammation and/or infection sites, oxygen consumption is elevated and can result in the interruption of the blood supply and the development of a hypoxic tissue microenvironment [3, 4]. Under such conditions, macrophage polarization towards inflammatory activities should be expected. Previous studies by our group and as well as this one showed that hypoxia modulates antileishmanial capacity of macrophages against *L. amazonensis* infection, reducing the percentage of infected cells and the number of intracellular parasites [7, 10]. In contrast, the extracellular growth of *L. amazonensis* promastigotes and amastigote and macrophage viabilities were not altered by hypoxia [this work and 7, 10], suggesting macrophage activation for the killing of parasites. The mechanisms by which macrophages control infection under hypoxia are not yet understood. Further experiments were conducted to elucidate whether the well-characterized defence of macrophages against intracellular micro-organisms might be involved in cell resistance under hypoxia. Nitric oxide is an important active component engaged in the killing of *Leishmania* in cytokine-activated macrophages [21, 22]. However, this work shows that knockout macrophages for iNOS expression lacking nitric oxide synthesis are still able to reduce infection under hypoxia, and our previous results indicated that hypoxia does not induce nitric oxide synthesis or iNOS expression infected macrophages [7]. Therefore, results do not suggest any correlation between nitric oxide and infection reduction under hypoxia. In addition to nitric oxide, cytokine activation of macrophages leads to ROS production through oxidative burst that is ultimately responsible for leishmanicidal activity [24–26, 31]. Interestingly, the antioxidants NAC, a thiol compound that act as direct ROS scavenger [16, 17], and ebselen, a compound that mimics glutathione peroxidase, a H_2O_2-detoxifying enzyme, [18, 19] inhibited the leishmanicidal effect of hypoxia. Our interpretation of these findings is that ROS produced by cell could be toxic to the parasites. In fact, cellular damage by ROS in response to hypoxia is supported by data showing that antioxidants are able to abrogate hepatocyte ROS-induced cell death during hypoxia [32]. Other recent study also showed that the blockade of endogenous GSH antioxidant synthesis inhibited glioma cells growth under hypoxic condition [33]. Because many highly reactive species such as singlet oxygen, superoxides, peroxides, hydroxyl radical, and hypochlorous acid are generated in cells during oxidative burst, participation of every ROS in parasite damage is difficult to define [34, 35].

Cytokines associated with Th1 and Th2 cells are important to determine resistance and susceptibility during leishmaniasis [13]. In this work, IL-10 and IL-12 releases were similar in infected macrophages under normoxia and hypoxia. Although hypoxia induced TNF-α production in non-stimulated and IFN-γ + LPS–stimulated macrophages, it did not alter TNF-α production in infected cells. Among the cytokines evaluated in this study, IL-6 was the only to present a small but significant increase in infected macrophages under hypoxia; however, the increase was significantly lower than IL-6 production after LPS stimulation. IL-6 is a proinflammatory cytokine,

which also exhibits pleiotropic effects on many cell types, and is a hypoxia-inducible molecule [36, 37]. As previous data have shown that IL-6 may inhibit human macrophages activated by IFN-γ, TNF-α, IL-3 and LPS for killing *L. amazonensis* [38], the current results could be explained by a non-causal effect of IL-6 on the macrophage leishmanicidal effect under hypoxia.

An alternative hypothesis that could explain the low *L. amazonensis* infection in the hypoxic condition is that parasite internalization by macrophages is damaged under hypoxia. Findings reported here show that the macrophage capacity for phagocytosis of fixed parasites was affected by hypoxia. These data are consistent with studies showing peritoneal macrophages with reduced uptake of latex beads, zymozan and zymocell and alveolar macrophages with reduced uptake of red blood cells under hypoxia [39, 40]. However, it appears that hypoxia did not affect live *L. amazonensis* entry into macrophages, indicating that parasite internalization is more complex than that of inert particles or dead parasites, because the process involves the action of both the host cell and the parasite [41]. The possibility that parasites might be released from macrophages under hypoxia, in a process of exocytosis, leading to diminished infection, was investigated because exocytotic release of red blood cells from alveolar macrophages under hypoxia was previously described [28]. Data analysis showed that hypoxia did not induce the exocytotic process of internalized particles by macrophages, and thus, it was concluded that impaired parasite internalization and exocytosis are not related to the antileishmanial activity of macrophages under hypoxia.

Although macrophages are one the types of cells that adapt to hypoxia by switching from aerobic to anaerobic glycolytic pathway for ATP production [42], glycolysis is inefficient, consuming 15 times more glucose per ATP molecule than oxidative phosphorylation [43]. These data persuaded us to analyse ATP production by macrophages to determine whether hypoxia induces energetic metabolism damage in cells that contribute to parasite death. The depletion of ATP occurred only in uninfected cells under severe hypoxia (1% O_2) as previously demonstrated [42–44]. However, infected cells produced similar levels of ATP when cultured under severe hypoxia (1% O_2), hypoxia (6% O_2) or normoxia (21% O_2) indicating that the reduction in the infection of macrophages in hypoxia is not related to any impairment of energetic metabolism.

Chemicals produced by macrophages, such as nitric oxide [24], and drugs treatment, such as miltefosine [45] and antimonials [46], induce an apoptosis-like death in *Leishmania* intracellular amastigotes. This work investigated whether apoptosis is induced in intracellular amastigotes by hypoxia. In contrast to IFN-γ + LPS–activated macrophages, which produce nitric oxide and induce apoptosis-like death in intracellular amastigotes [29], infected macrophages under hypoxia showed no intracellular parasites with DNA fragmentation when analysed by the TUNEL assay. These results led to the conclusion that apoptosis-like death does not occur in intracellular parasites within macrophages under hypoxia.

Our group previously demonstrated that infection with *L. amazonensis* results in HIF-1α expression in a murine model of disease and *in vitro* [10, 11] and, more recently, HIF-1α activation was detected in human skin biopsy of patient suffering of leishmaniasis [47]. The infection of macrophages with the parasite was not associated with cellular hypoxia, as confirmed by the hypoxia marker pimonidazole, suggesting an oxygen-independent activation of HIF-1α [10]. This report showed, for the first time, that HIF-2α, which despite extensive sequence homology with HIF-1α has no overlapping roles and distinct spatial-temporal patterns of expression [48, 49], is upregulated during an intracellular infection. The *L. amazonensis* and *Toxoplasma gondii* survival was reduced in cells lacking HIF-1α expression [10, 50] suggesting that HIFs could be part of an adaptative mechanism to maintain parasitized cell and parasite integrity; however, further studies are required to clarify this point.

To summarize, analysis of the data indicated that hypoxia induces macrophages to control *L. amazonensis* infection, and nitric oxide, TNF-α, IL-10 or IL-12 are not responsible for the decrease in parasitism. Live parasite entry or exocytosis of internalized particles, as well as energetic metabolism, were not impaired in infected cells under hypoxia; no apoptosis-like death was detected in intracellular parasites. ROS is likely to be involved, because treatment with antioxidants NAC and ebselen inhibits the leishmanicidal effect of cells under hypoxia. *Leishmania amazonensis* infection induces macrophages to express hypoxia-inducible factor-1 (HIF-1α) and -2 (HIF-2α). Furthermore, these results indicate that hypoxia, a microenvironmental factor present in infected tissues, affects the microbial activities and protein expression of macrophages leading to a different phenotype from that of the normoxic and that it has a role in modulating *Leishmania* infection.

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Authors' contribution

AD, MCC and WWA-S carried out the design and the experiments, PPJ and FRG participated in the analysis of results, and SG conceived of the study and wrote the manuscript.

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