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HIF-1α-regulated MIF activation and Nox2-dependent ROS generation promote *Leishmania amazonensis* killing by macrophages under hypoxia

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ABSTRACT

Increasing attention is given to the finding that macrophages under hypoxia are capable of controlling infection by the intracellular protozoan parasite *Leishmania amazonensis*. The hypoxia-inducible factor (HIF)-1α has been shown to play an essential role in this enhanced innate immune response. Our study aimed to explore the HIF-1α-dependent mechanisms leading to reduced survival of the parasites residing in macrophages under low oxygen conditions. Hypoxia triggered ($P < 0.01$) NADPH oxidase 2 (Nox2) expression and reactive oxygen species (ROS) production in J774 macrophages upon 24-h infection with *L. amazonensis*. Furthermore, increased ($P < 0.01$) expression levels of HIF-1α and macrophage migration inhibitory factor (MIF) were detected in the infected cells grown at 3% oxygen tension. We found that either HIF-1α silencing, Nox2 inhibition or MIF antagonism caused a significant ($P < 0.05$) reversal of the improved leishmanicidal activity displayed by the hypoxic phagocytes. Taken together, our current results suggest that, under conditions of limited availability of oxygen, activation of the HIF-1α/MIF axis via Nox2/ROS induction promotes killing of *L. amazonensis* amastigotes by macrophages. Such protective mechanism might operate in *L. amazonensis*-infected tissues where low oxygen levels prevail.

1. Introduction

Hypoxia plays a critical role in the pathobiology of inflamed, diseased tissues, including multiple sites of infection with an array of microbes [1–3]. The influx of macrophages into affected areas augments notably with the onset and progression of infection, displaying pronounced accumulation in avascular and necrotic zones, where they are exposed to low oxygen tension. Under such conditions, these cells respond rapidly by altering their patterns of gene expression, cytokine secretion, display of surface antigens, migration and/or phagocytic activity [4,5]. Increasing attention is given to the finding that hypoxia may be beneficial in terms of reducing the development of infection [6]. Particularly, growing evidence suggests that limited availability of oxygen enables macrophages to control infection by *Leishmania amazonensis*, an intracellular protozoan parasite responsible for cutaneous, diffuse and mucocutaneous leishmaniasis [7–10]. Macrophages are key players in early generation of proinflammatory mediators and elimination of Leishmania amastigotes. Arrais-Silva and colleagues [11] demonstrated that macrophages in the hypoxic microenvironment of skin lesions from *L. amazonensis*-infected mice express the hypoxia-inducible factor (HIF)-1α. This transcription factor has been associated with innate antileishmanial immune responses [12]. However, HIF-1α-dependent mechanisms by which macrophages could control *L. amazonensis* infection under hypoxia are not fully understood yet. Our current goal was to examine whether induction of the cytokine macrophage migration inhibitory factor (MIF), susceptible to up-regulation by HIF-1α signaling and depressed oxygen tension [13], contributes to reduced intracellular survival of *L. amazonensis* in macrophages under low oxygen conditions.

Abbreviations: HIF-1α, hypoxia-inducible factor-1α; MIF, macrophage migration inhibitory factor; siRNA, small interfering ribonucleic acid; DPI, diphenyleneiodonium chloride; ISO-1, (S, R)-3-(4-hydroxyphenyl)-4, 5-dihydro-5-isoxazole acetic acid methyl ester; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; Nox2, isoform 2 of NADPH oxidase

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2. Materials and methods

2.1. Leishmania infection of J774 cells

Leishmania amazonensis (MHOM/VE/84/MEL) amastigotes were isolated from active skin lesions of BALB/c mice as described previously [14]. Viable parasites were suspended in RPMI medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, 100 mM HEPES, and 10% fetal calf serum (FCS) and used immediately after isolation.

The murine macrophage cell line J774 obtained from the American Type Culture Collection (Manassas, VA, USA) was maintained in RPMI supplemented with antibiotics and 10% FCS at 37 °C in a humidified incubator with 21% O_2, 5% CO_2 and balanced N_2. J774 macrophages were infected by adding to the cell cultures a suspension of *L. amazonensis* amastigotes in RPMI supplemented with antibiotics at a 10:1 parasite-to-cell ratio. One hour later, cultures were washed to remove extracellular parasites and replaced with fresh medium for additional 24 h under conditions of either normoxia [21% (150 mm Hg) oxygen tension] or hypoxia [3% (22 mm Hg) oxygen tension]. Hypoxic cell culture conditions were established as described [10]. Intracellular Leishmania destruction was assessed by evaluation of the percentage of infected macrophages and the number of amastigotes per macrophage. For this, the cells on coverslips were stained with Giemsa. The amastigotes, which are exclusively localised in parasitophorous vacuoles within the phagocytes, were examined microscopically at ×1000 magnification [15]. At least 700 cells were counted per triplicate coverslip.

Primary murine macrophages were collected from the peritoneal cavities of male BALB/c mice elicited with 2 mL of thioglycolate medium five days before the experiments. Mice were obtained from Charles River Laboratories (Wilmington, MA, USA) at five weeks of age. All mice were housed in standard environmental conditions and fed with a pellet diet and water *ad libitum*. Murine experiments were conducted using protocols approved by the Animal Care and Use Committee (number 0159-12-17) of the National University (Trujillo, Peru).

2.2. Inhibition assays

In some experiments, J774 macrophages were transfected with mouse specific small interfering RNA (HIF-1α siRNA, Santa Cruz Biotechnology, Dallas, TX, USA) following a protocol that has been proved to efficiently silence gene expression of this factor [16]. A negative control consisting of macrophages transfected with scrambled siRNA (Cell Signaling, Danvers, MA, USA) was included in the analysis. Cells were used for infection and analysis of MIF production two days later. Also, selective inhibition of NADPH oxidase 2-dependent generation of reactive oxygen intermediates (using 1 μM diphenyleneiodonium chloride -DPI-, Sigma-Aldrich, St. Louis, MO, USA) or specific MIF antagonism (using 50 μM [(S, R)-3-(4-hydroxyphenyl)-4, 5-dihydro-5-isoxazole acetic acid methyl ester] -ISO-1-, EMD Millipore, Billerica, MA, USA) was assayed in Leishmania-infected macrophage cultures.

2.3. Quantification of intracellular levels of reactive oxygen species (ROS)

ROS generation was measured by the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-Aldrich) fluorescence method [17]. Briefly, J774 macrophages (10^6) were washed, suspended in 1 ml of phosphate-buffered saline (PBS) and incubated with 100 μM DCFH-DA for 30 min at 37 °C. The cells were then infected with *L. amazonensis* for 1 h at 37 °C with 21% O_2, and for additional 24 h under normal (21%) or low (3%) oxygen tension. Uninfected cultures were included as controls for each condition. Macrophages were then formalin-fixed for 15 min, harvested, repeatedly washed and analysed by flow cytometry at a wavelength of 488 nm. Samples were acquired on a Sysmex Partec (Görlitz, Germany) PAS-*III* flow cytometer and the data were analysed using Cyflogic 1.2.1 software. Results are expressed as percentage of DCFH positive cells.

2.4. Western blotting

J774 cell samples were disrupted and solubilized extracts (20–30 μg) were separated in SDS-PAGE gels (10% acrylamide), and then transferred to a nitrocellulose membrane (EMD Millipore). After blocking for 2 h with 5% non-fat dried milk in Tris-buffered saline containing 0.1% Tween-20, the membrane was probed overnight at 4 °C with specific antibodies against mouse NADPH oxidase 2 (Nox2, Abcam, Cambridge, UK), HIF-1α (Cayman, Ann Arbor, MI, USA) or α-tubulin (Thermo Fisher Scientific, Waltham, MA, USA). A HRP-conjugated appropriate secondary antibody was dispensed to detect immunoreactive bands using an ECL system (GE Healthcare, Pittsburgh, PA, USA). Band intensity was analysed using NIH Image J software.

2.5. Measurement of mouse macrophage migration inhibitory factor (MIF)

J774 macrophages were infected for 24 h with *L. amazonensis* amastigotes under normoxic or hypoxic condition. The media were then harvested and used to determine MIF level by sandwich ELISA (Kamiya Biomedical, Seattle, WA, USA) according to the manufacturer's instructions. The supplied standard was used to generate the standard curve. The assay sensitivity was 0.16 ng/ml.

2.6. Apoptosis assay

Apoptotic cells were identified by annexin V (AV) and propidium iodide staining using ApoAlert Annexin V-FITC apoptosis kit (Takara Bio, Mountain View, CA, USA) following the manufacturer's instructions. FACS analysis was performed on an EPICS Elite *ESP* flow cytometer (Beckman Coulter, Brea, CA, USA) with an argon-ion laser tuned to 488 nm. The apoptotic cells were estimated as the percentage of macrophages that stained positive for AV while remaining impermeable or permeable to PI (early or late apoptosis, respectively). Viable cells were both AV and PI negative.

2.7. Statistical analysis

Data analysis was carried out using Prism 5.0 software (GraphPad Software, San Diego, CA, USA). Arithmetic means and standard deviations (SD), derived from at least triplicate observations per sample, were calculated. The results are expressed as the mean ± SD. Mann-Whitney *U* test and one-way analysis of variance (ANOVA) followed by a *post-hoc* Tukey test were used to determine the statistical significance of the intergroup comparisons. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Hypoxia promotes leishmanicidal activity in J774 macrophages

In a first step, *L. amazonensis* amastigotes were allowed to invade murine macrophages of the J774 cell line for 1 h. The infected cells were then washed and incubated for additional 24 h under conditions of either normoxia (21% oxygen tension) or hypoxia (3% oxygen tension). Fig. 1 displays parasite load within J774 macrophages. After 24 h of infected culture exposed to hypoxia, we observed a significant ($P < 0.05$) reduction in the percentage of Leishmania-harboring macrophages (22.6 ± 5.4% vs 53.1 ± 9.8%, Fig. 1A) and also in the number of amastigotes per cell (2.8 ± 1.0 vs 5.9 ± 1.1 intracellular parasites/phagocyte, Fig. 1B), compared with infected cultures maintained under normal oxygen tension. No changes in macrophage

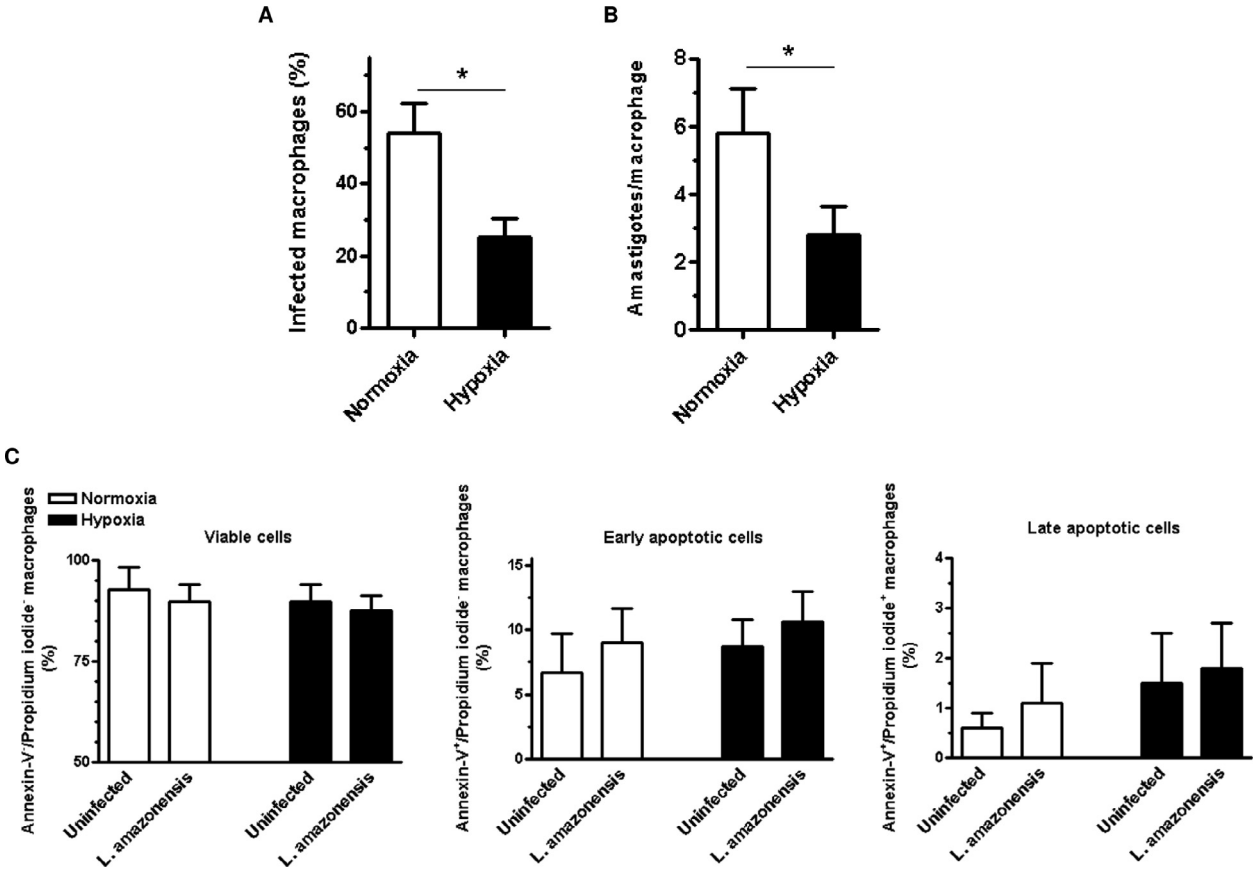

Fig. 1. Effect of hypoxia on *Leishmania amazonensis* infection of murine J774 macrophages. Under normoxic conditions, the phagocytic cells were infected with *L. amazonensis* amastigotes for 1 h. Cell cultures were washed and incubated for an additional 24 h in normoxia or hypoxia. Intracellular Leishmania killing was assessed by evaluation of the percentage of infected macrophages (A) and the number of amastigotes per macrophage (B) as described under Materials and methods. (C) Bar diagram shows apoptosis in hypoxic and normoxic J774 cells, uninfected or infected for 24 h with *L. amazonensis* parasites. Flow cytometry analysis was performed as indicated in the Materials and methods section. The apoptotic cells were estimated as the percentage of macrophages that stained positive for annexin V (AV) while remaining impermeable (early apoptosis, middle) or permeable (late apoptosis, right) to propidium iodide (PI). Viable cells (left) were both AV and PI negative. The results represent the mean ± SD of three independent experiments, each performed in triplicate. The significance of the difference between cell cultures in hypoxia and normoxia is indicated in the figure. $*P < 0.05$.

viability and proliferation were found in uninfected J774 cultures grown in normoxic and hypoxic microenvironment. *In vitro* infection with *L. amazonensis* has been reported to trigger apoptosis of murine macrophages [18]. In our hands, Leishmania infection led to a slight shift in the percentage of apoptotic J774 cells, under either normoxia or hypoxia, but the differences observed were not statistically significant [6.7 ± 3.0% for uninfected cells vs 9.1 ± 2.6% for infected cells under normoxic conditions (P = 0.37) and 8.6 ± 2.1% for uninfected cells vs 10.7 ± 2.3% for infected cells under hypoxic conditions (P = 0.33) in the macrophage subpopulation that underwent early apoptosis; 0.6 ± 0.3% for uninfected cells vs 1.1 ± 0.8% for infected cells under normoxic conditions (P = 0.36) and 1.5 ± 1.0% for uninfected cells vs 1.8 ± 0.9% for infected cells under hypoxic conditions (P = 0.72) in the macrophage subpopulation that underwent late apoptosis; Fig. 1C]. Collectively, these data indicate that hypoxia can markedly enhance macrophage resistance to *L. amazonensis* infection.

3.2. *L. amazonensis-infected macrophages under hypoxia develop an oxidative stress response*

ROS generation is considered to be a major macrophage effector mechanism in cutaneous leishmaniasis [19]. Consequently, we next asked whether hypoxia could boost ROS levels upon *in vitro* infection of J774 cells. Under conditions of normoxia, the cells exposed to the parasite for 24 h elicited an oxidative response increasing from 5.2 ± 0.7 to 15.4 ± 1.6% of $DCFH^+$ cells ($P < 0.05$) when compared to that detected in the uninfected control (Fig. 2A). Infection under low oxygen tension drove an enhanced respiratory burst in parasite-harboring phagocytes. The combined effect of L. amazonensis and hypoxia led to significantly ($P < 0.01$) augmented ROS formation in macrophages (17.7 ± 1.4 vs 33.7 ± 2.9% of $DCFH^+$ cells for uninfected and infected cultures, respectively). Remarkably, Leishmania-dependent induction of oxidative stress under hypoxia was even greater than that achieved under 21% oxygen tension (15.4 ± 1.6 vs 33.7 ± 2.9% of $DCFH^+$ cells for normoxic and hypoxic infected cultures, respectively; $P < 0.01$). These results suggest that hypoxia potentiates ROS production upon macrophage invasion by *L. amazonensis* amastigotes.

To analyse the mechanism underlying ROS accumulation in infected cells subjected to low oxygen tension, we further verified the induction of the Nox2 isoform of NADPH oxidase by Western immunoblotting. As shown in Fig. 2B, *L. amazonensis* infection under normoxia triggers a weak, albeit significant ($P < 0.05$), elevation of 58-KDa Nox2 protein content in J774 macrophages. Hypoxia stimulated Nox2 levels in parasite-free cultures and led to nearly a three-fold ($P < 0.01$) increased expression of this ROS-generating enzyme after 24 h of infection. These observations show that hypoxia exerts strong positive effect on Nox2 expression in the infected macrophage.

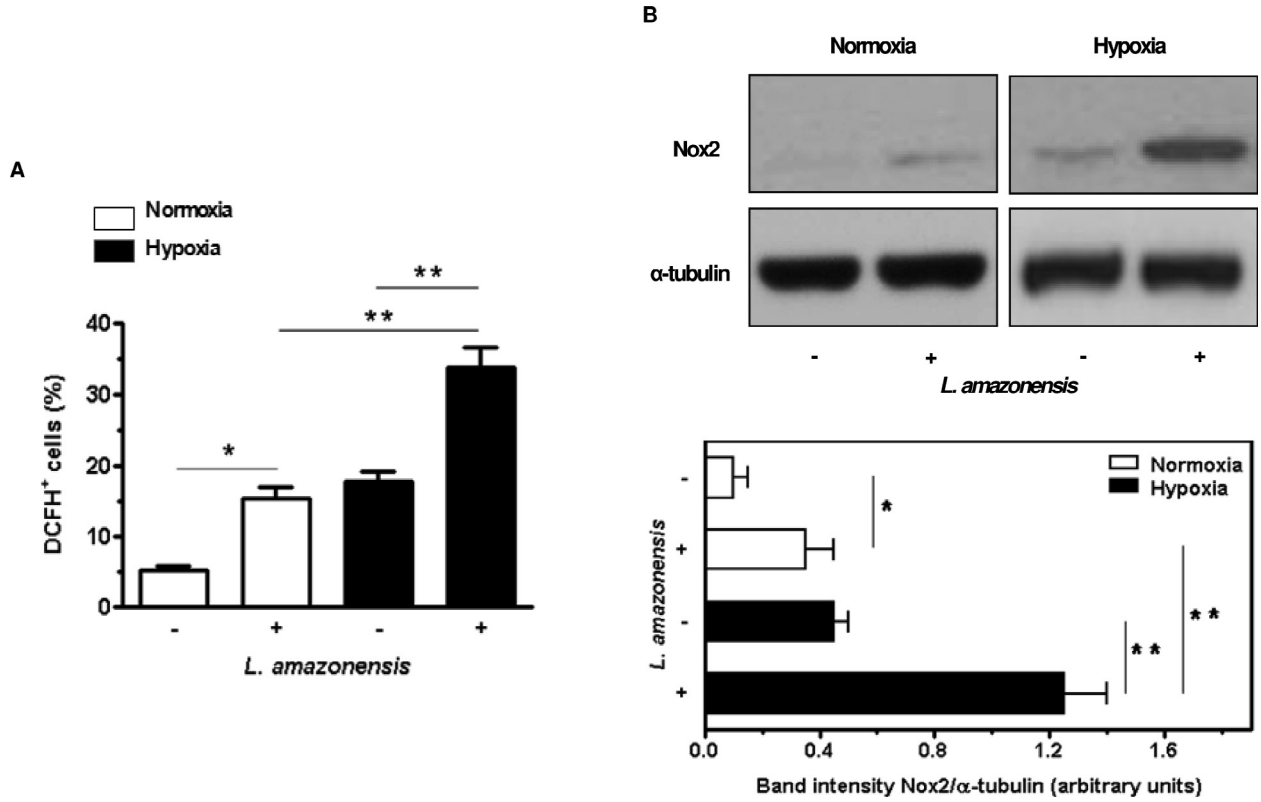

Fig. 2. NADPH oxidase isoform 2-dependent release of reactive oxygen species in J774 macrophages infected with *Leishmania amazonensis* under normoxia or hypoxia. J774 macrophages were infected (+) with Leishmania amastigotes and exposed to 21% or 3% oxygen tension. Uninfected cells (−) were used as controls for each condition. Cell specimens and culture supernatants were collected at 24 h of infection. (A) Quantification of ROS generation by the DCFH-DA fluorescence procedure as described under Materials and methods. Values are expressed as percentage of DCFH positive cells. (B) Immunoblot analysis of Nox2 induction. Alpha-tubulin was used as a loading marker. Band intensity was analysed using NIH Image J software. All results shown represent means ± SD of three individual experiments assayed in triplicate. *$P < 0.05$; **$P < 0.01$.

3.3. Nox2-generated ROS signal favors HIF-1α-dependent MIF production by Leishmania-infected macrophages under hypoxia

We next addressed whether augmented levels of Nox2 and ROS in the infected phagocytes under low oxygen tension contribute to activate the HIF-1α/MIF axis in J774 cells. In comparison with uninfected controls, macrophages infected with *L. amazonensis* amastigotes for 24 h under hypoxia, but not under normoxia, demonstrated significantly ($P < 0.01$) increased HIF-1α protein content (Fig. 3A). This stimulatory effect was abolished ($P < 0.01$) by NADPH oxidase inhibition with diphenyleneiodonium (DPI). Furthermore, we detected by ELISA heightened ($P < 0.05$) production of MIF in response to 24-h parasite infection under normoxia that was greatly enhanced ($P < 0.01$) in the infected hypoxic cells (Fig. 3B). We also examined the effect of HIF-1α silencing on the release of this proinflammatory cytokine (Fig. 3B and Supplementary Fig. 1). Inhibiting HIF-1α expression with specific siRNA was sufficient to markedly ($P < 0.05$) attenuate MIF induction cooperatively triggered by *L. amazonensis* and 3% oxygen tension. A similar outcome was achieved in Leishmania-infected peritoneal macrophages isolated from naïve BALB/c mice (Fig. 3, right panels). These results suggest that parasite-elicited MIF expression in hypoxic J774 cells is up-regulated via the Nox2/ROS/HIF-1α pathway.

3.4. HIF-1α-promoted MIF activation and Nox2-dependent ROS generation foster L. amazonensis killing by macrophages under hypoxia

In an attempt to verify that activation of the HIF-1α/MIF axis and Nox2/ROS induction effectively exerted lethal effects on intracellular *L. amazonensis* forms, amastigote load was determined in J774 cells infected under hypoxia, in the absence or presence of negative regulators of each of the above pathways. Abolishing Nox2-dependent ROS formation by addition of DPI resulted in a substantial ($P < 0.05$) elevation in the percentage of parasitised macrophages (Fig. 4A) and the number of amastigotes per cell (Fig. 4B) compared to those recorded for phagocytes devoid of inhibitory treatment. Likewise, we found that either HIF-1α silencing or MIF antagonism by ISO-1 (Supplementary Fig. 1) caused a significant ($P < 0.05$) reversal of the microbicidal activity displayed by macrophages infected under low oxygen tension without adding any inhibitor (Fig. 4A, B). No synergistic interaction was demonstrated for the combination of DPI, HIF-1α siRNA and ISO-1. Altogether, these findings suggest that Nox2, ROS, HIF-1α and MIF are clearly implicated in controlling multiplication of *L. amazonensis* amastigotes inside hypoxic macrophages.

4. Discussion

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. In the mouse model of infection with *L. amazonensis*, low oxygen levels have been detected in cutaneous lesions [20]. More interesting, several reports have revealed the strong influence of tissue hypoxia on macrophage response to Leishmania infection [8,10,12]. Limited availability of oxygen can modulate macrophage protein expression and functional activity, resulting in altered susceptibility to intracellular infection with the parasite [5,10]. In agreement with previous data [8], we observed that hypoxia enhances macrophage resistance to *L. amazonensis* infection *in vitro*. J774 macrophages exposed to low oxygen tension showed a significant reduction in the percentage of infected cells and the number of amastigotes per cell. We established a 3% oxygen tension in

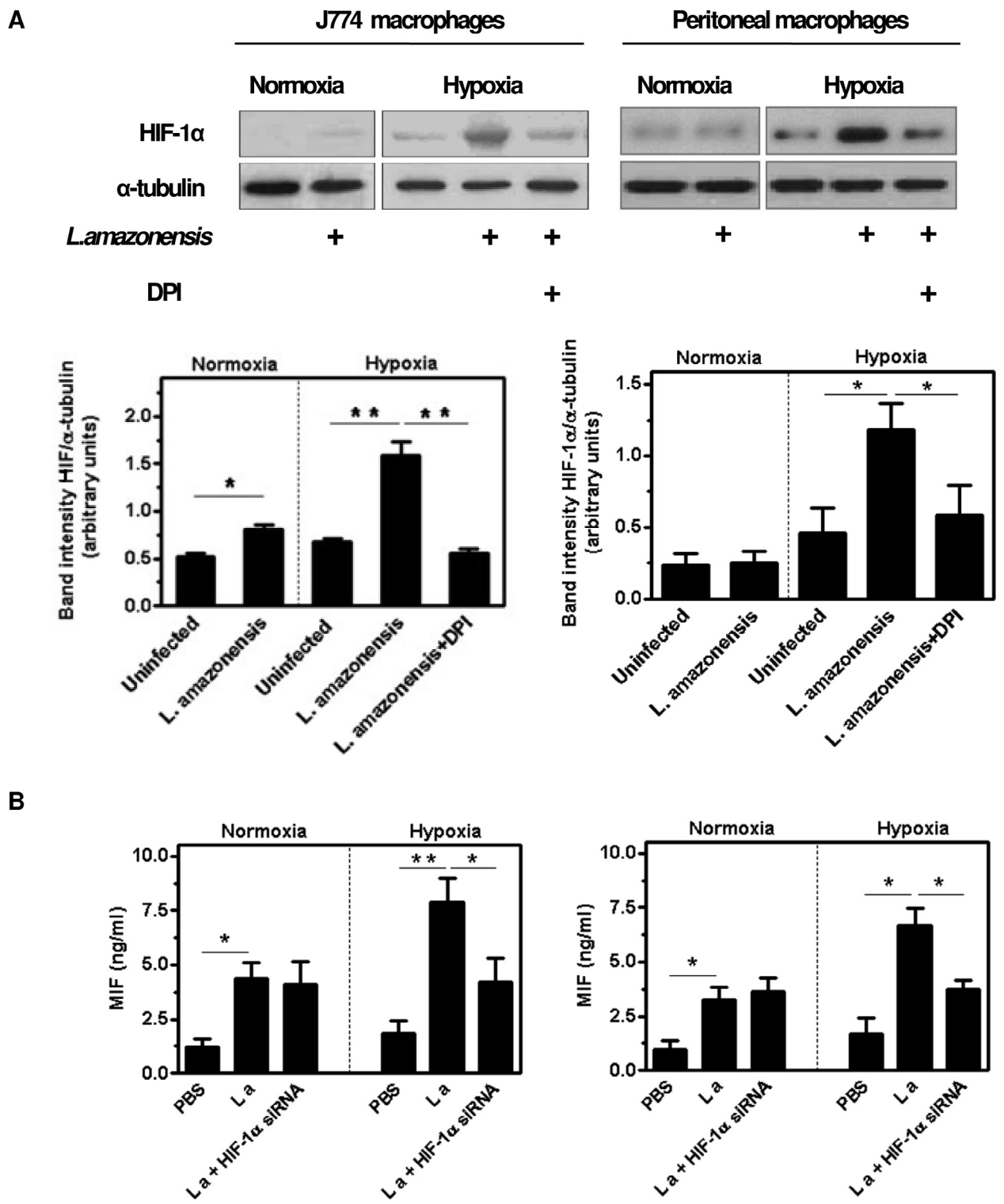

Fig. 3. Effect of *Leishmania amazonensis* infection of macrophages exposed to normal and low oxygen tension on the induction of hypoxia-inducible factor-1α and macrophage migration inhibitory factor. J774 cells (left panels) or mouse peritoneal macrophages (right panels) were infected with the parasite for 24 h under normoxia or hypoxia. Uninfected (PBS) samples were also assayed. In a set of experiments, macrophage cultures were treated for 30 min before infection with a selective inhibitor of the isoform 2 of NADPH oxidase (1 μM diphenyleneiodonium chloride, DPI) or transfected with small interfering RNA for HIF-1α silencing (HIF-1α siRNA). (A) The expression levels of HIF-1α protein were analysed by immunoblotting. Quantification of blot signals was accomplished by densitometry using α-tubulin levels as a control. Results show a representative experiment of three performed in triplicate. $^{**}P < 0.01$. (B) MIF concentration in 24-h culture supernatants was quantified by ELISA. L a, *Leishmania amazonensis* infection. Each bar represents the mean ± SD of triplicate determinations for the corresponding condition. Results were reproduced in three independent experiments. Statistically significant differences are indicated ($^{*}P < 0.05$; $^{**}P < 0.01$).

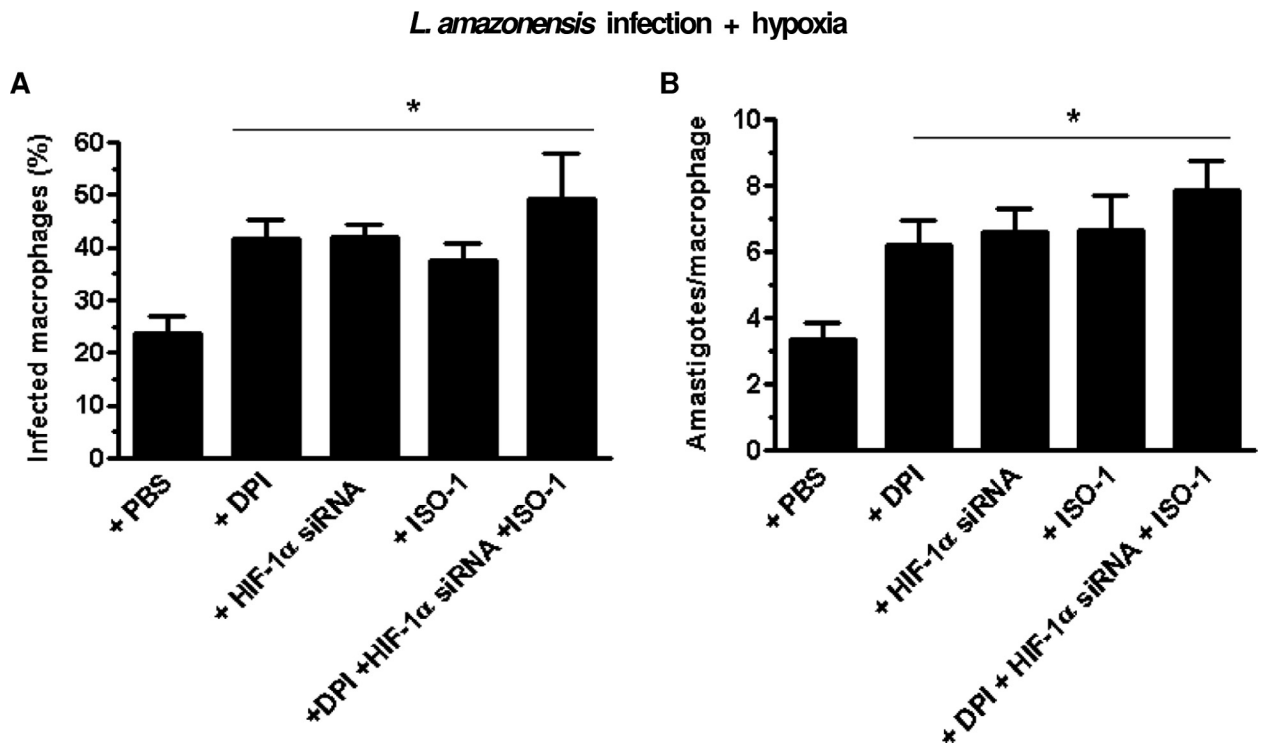

Fig. 4. Involvement of macrophage-derived factors in leishmanicidal activity of J774 cells infected under hypoxia. J774 macrophages were infected with *L. amazonensis* amastigotes under conditions of hypoxia (3% oxygen tension). In a set of experiments, the phagocytes were incubated for 30 min before infection with selective inhibitors of NADPH oxidase 2 (1 μM DPI) or MIF (50 μM [(S, R)-3-(4-hydroxyphenyl)-4, 5-dihydro-5-isoxazole acetic acid methyl ester], ISO-1), or transfected with HIF-1α siRNA, or treated with a combination of the three antagonists. The control of *L. amazonensis* amastigote growth within hypoxic J774 cells was assessed by evaluation of the percentage of infected macrophages (A) and the number of amastigotes per macrophage (B) at 24 h postinfection as described under Materials and methods. Values represent the mean ± SD of three independent experiments, each performed in triplicate. $^*P < 0.05$.

the culture medium under hypoxic conditions akin to that (~2.8%) recently reported by Mahnke and colleagues [21] for leishmanial skin lesions. Hypoxia-triggered parasiticidal activity has also been documented in *L. amazonensis*-infected peritoneal macrophages and found to be unrelated to apoptosis induction, impaired parasite internalisation/exocytosis or energetic metabolism damage [10].

The mechanisms by which macrophages are capable of controlling *L. amazonensis* infection during hypoxia are largely unknown. Different studies suggest that nitric oxide, an important mediator engaged in amastigote killing by activated macrophages, is not a major responsible for the decrease in intracellular parasite load under hypoxia [8,10]. Nevertheless, ROS involvement has been suspected, since a ROS scavenger and a glutathione peroxidase mimetic effectively inhibited the leishmanicidal activity of macrophages under low oxygen tension [10]. ROS release through oxidative burst is another relevant effector function potentiating antileishmanial defense in mononuclear phagocytes [22]. Our findings indicate that hypoxia induces Nox2 expression and ROS formation in infected macrophages that may improve clearance of amastigotes. There is perhaps an apparent paradox in augmented ROS generation in a cell microenvironment lacking O_2 as a substrate. However, a possible explanation for this is to be found in current concepts that provide evidence for a complex interaction between a hypoxic mitochondrial biology and the ability to produce ROS. Mitochondria are severely affected by decreased oxygen availability. As a consequence, different adaptive changes occur in the organelle morphology and metabolism, resulting in modified composition/assembly of subunits of the respiratory chain, oxidative phosphorylation and reductive carboxylation, all of which allow ROS increment even at depressed oxygen levels [23].

In the J774 cell line, Leishmania infection promotes HIF-1α activation by dual mechanism involving transcriptional up-regulation as well as simultaneous stabilization of the factor through parasite-dependent prolyl hydroxylase blocking activity. Even more important, intracellular amastigotes can exploit HIF-1α induction as a survival strategy inside host phagocytes [24]. Macrophages in the hypoxic microenvironment of leishmanial lesions have been shown to overexpress HIF-1α, mostly in the cytoplasm and surrounding the amastigotes inside the parasitophorous vacuoles [11,19,25]. Coincidently, we demonstrated increased expression of this mediator in J774 cells infected with *L. amazonensis* under hypoxia. Decreased oxygen tension is known to induce the production of HIF-1α that requires further stabilization by Nox2-dependent ROS activity [10,16,26]. Such response could represent another adaptive mechanism aimed to keep the integrity of a parasitised cell devoid of oxygen [23]. Complex III of the mitochondrial electron transport chain is involved in hypoxia-induced free radical production that favors HIF-1α stabilization [26]. Several signal transduction pathways have been associated with this process. For example, Paik and colleagues [27] presented evidence for a protein kinase C (PKC)-dependent increase in ROS and HIF-1α levels during hypoxia. In Leishmania-containing macrophages, parasite infection modulates intracellular PKC signaling resulting in impaired assembly of Nox complex, which is responsible for superoxide anion generation and hence amastigote killing [28]. The invading pathogen differentially affects PKCα, βI, βII, and ε isoforms but enhances the expression, phosphorylation and translocation of the PKCδ, ζ, and λ isoforms that mediate resistance or susceptibility to infection by regulating the mitochondrial-ROS response in macrophages [29].

Hypoxia provokes changes in gene expression that are orchestrated mainly by the transcriptional activator HIF-1α. It should be noted that the proinflammatory cytokine MIF has been identified as a target gene of HIF-1α in oxygen-depleted conditions [13] and also detected in cutaneous lesions of mice with leishmaniasis [20]. In fact, a functional MIF/HIF-1α interplay has been observed in cancer cells [30]. This pleiotropic cytokine is known to be protective against Leishmanial

infection. MIF appears to be capable of killing amastigotes within macrophages by acting directly or indirectly with other cytokines and reactive oxygen and nitrogen intermediates [31]. Accordingly, mice genetically deficient for MIF are more susceptible to Leishmania infection because of impaired macrophage leishmanicidal activity associated with low superoxide and nitric oxide production [32]. Furthermore, MIF polymorphism has been shown to favor infection and disease progression in cutaneous leishmaniasis patients [33].

To the best of our knowledge, our study is the first report describing MIF-mediated killing of intracellular forms of *L. amazonensis* by hypoxic mononuclear phagocytes. Destruction of amastigotes of Leishmania species other than *L. amazonensis* by ROS and MIF derived from hypoxic macrophages is currently under investigation. In contrast to *L. major*, which is able to adapt to hypoxia [34], *L. amazonensis* survival is evidently affected in macrophages exposed to 3% oxygen tension. Hypothetically, different susceptibilities to NO-independent antileishmanial activity and/or O_2 demand of *L. amazonensis* and *L. major* might explain the divergent outcome of macrophage infection by the respective Leishmania species in a low oxygen microenvironment [12], but further studies are required to assess these issues. To summarize, our current findings suggest that, under conditions of limited availability of oxygen, activation of the HIF-1α/MIF axis via Nox2/ROS induction exerts parasiticidal effects on *L. amazonensis* amastigotes residing inside J774 macrophages. Whether such a mechanism observed *in vitro* plays a major role in the first line of cutaneous host defense against Leishmania infection is a question to be addressed.

5. Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2018.10.007.

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