
Hypoxia modulates phenotype, inflammatory response, and leishmanial infection of human dendritic cells

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Development of hypoxic areas occurs during infectious and inflammatory processes and dendritic cells (DCs) are involved in both innate and adaptive immunity in diseased tissues. Our group previously reported that macrophages exposed to hypoxia were infected with the intracellular parasite *Leishmania amazonensis*, but showed reduced susceptibility to the parasite. This study shows that although hypoxia did not alter human DC viability, it significantly altered phenotypic and functional characteristics. The expression of CD1a, CD80, and CD86 was significantly reduced in DCs exposed to hypoxia, whereas CD11c, CD14, CD123, CD49 and HLA-DR expression remained unaltered in DCs cultured in hypoxia or normoxia. DC secretion of IL-12p70, the bioactive interleukin-12 (IL-12), a cytokine produced in response to inflammatory mediators, was enhanced under hypoxia. In addition, phagocytic activity (*Leishmania* uptake) was not impaired under hypoxia, although this microenvironment induced infected DCs to reduce parasite survival, consequently controlling the infection rate. All these data support the notion that a hypoxic microenvironment promotes selective pressure on DCs to assume a phenotype characterized by pro-inflammatory and microbial activities in injured or inflamed tissues and contribute to the innate immune response.

Key words: Dendritic cells; hypoxia; interleukin-12; leishmaniasis; *Leishmania amazonensis*.

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Inflammatory processes that occur in a wide variety of pathologic conditions, such as atherosclerotic disease, dermal wounds, cardiovascular disorders, and microbial infections, are implicated in the formation of hypoxic areas in tissues (1–6). Recently, our group showed that hypoxia-inducible factor-1 α (HIF-1 α), a regulator of the genetic response to hypoxia, is expressed in cutaneous lesions of mice infected with *Leishmania amazonensis*, a parasite of macrophages and dendritic cells (DCs) and one of the causative agents of cutaneous and diffuse

leishmaniasis in the Americas (7, 8). In addition, our studies demonstrated that experimental hypoxia induced macrophages to reduce intracellular parasitism, suggesting that hypoxia is important during *Leishmania* infection (9–11). Of relevance to *Leishmania* infection, evidence shows that DCs are involved in the persistence of the parasite, disease progression, and the establishment of T-cell-mediated immune response depending on several factors, including the *Leishmania* species, development stage, and exogenous DC stimuli (12–14). In addition, recent investigations have demonstrated phenotypic changes and alterations in the migration capacity of DCs under hypoxic conditions

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(15–24). Hence, investigating whether low oxygen tension alters human DC susceptibility to the intracellular parasite *Leishmania*, phenotype, and inflammatory responses is important to understand DC reaction to the microenvironment. In this study, phenotypic change, IL-12 production, and *Leishmania* susceptibility of human DCs cultured under low oxygen tension were evaluated.

MATERIALS AND METHODS

Parasites

Leishmania amazonensis (MHOM/BR/73/M2269) amastigotes were isolated from footpad lesions of BALB/c mice (Centro Multidisciplinar para Investigação Biológica, Unicamp, Campinas, São Paulo State, Brazil), as described previously elsewhere, and used immediately (9). All animal experiments were conducted in accordance with Institutional guidelines in compliance with the recommendations of the Guide for Care and Use of Laboratory Animals. The experimental protocol was approved by Universidade Estadual de Campinas' Animal Ethics Committee.

Generation of DCs from human blood monocytes

The generation of DCs from peripheral blood monocytes, drawn with informed consent from healthy donors, was performed as previously described elsewhere (25, 26). DCs were cultured in 24-well plates with coverslips or in 25 cm² culture flasks for 7 days in Iscove's medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with interleukin-4 (IL-4; 250 ng/ml) and granulocyte-macrophage colony-stimulating factor (GM-CSF; 50 ng/ml) (Sigma-Aldrich).

Cell culture conditions

Dendritic cells generated after 7 days at atmospheric oxygen levels (21% O₂, normoxia) in a humidified standard incubator at 37 °C were subsequently exposed for 24–72 h to an atmosphere of 5% O₂ (hypoxia) inside a gas-tight modular chamber (Billups-Rothenberg, Del Mar, CA, USA) or 21% O₂ atmosphere. The establishment of low oxygen tension conditions and gas-phase and culture media-phase oxygen measurement were described previously elsewhere (9–11). Cell viability was tested by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) formazan production, a colorimetric dye-reduction assay (Sigma-Aldrich). In experiments involving DC activation, the cells were treated with 100 ng/ml human γ -interferon (IFN- γ) and

100 ng/ml *Escherichia coli* lipopolysaccharides (LPS; Sigma-Aldrich) (28).

Cytometric analyses

Dendritic cells were stained with monoclonal antibodies to CD1a, CD11c, CD14, CD80, CD83, CD86, HLA-DR, and lineage cocktail CD3, CD16, CD19, and CD56 (Becton Dickinson, San Jose, CA, USA) as previously described elsewhere (29). Mouse IgG1 and IgG2 were used as controls. All antibodies were conjugated with fluorescein, phycoerythrin, or peridinin chlorophyll protein. Fluorescence-activated cell sorting (FACS) analyses were performed with a FACSort instrument and Cell Quest software.

Infection of DCs with *Leishmania*

Dendritic cells were infected with *L. amazonensis* amastigotes at a parasite–DC ratio of 3:1, as previously described elsewhere (25). After the period of infection, cells on the coverslips were stained with Giemsa and examined microscopically at 1000 \times magnification to evaluate the infection index (percentage of infected macrophages \times number of amastigotes per macrophage) as previously described elsewhere (25).

IL-12 quantification

Interleukin-12 was detected as a secreted protein product in culture supernatants using IL-12p70-specific ELISA (BD Biosciences Pharmingen, San Diego, CA, USA). The assays were performed according to the manufacturer's guidelines.

Data analyses

All experiments were repeated at least three times and the results are expressed as mean \pm SD. Statistical analyses were performed using the Student's *t*-test.

RESULTS

Our initial interest was to determine whether DCs exposed to two different oxygen tensions, 21% O₂ (normoxia) and 5% O₂ (hypoxia), would show comparable expression of DC-associated markers. Phenotype analysis was performed on DCs that were generated in GM-CSF and IL-4 for 7 days under normoxia and then incubated under normoxia or hypoxia for 24 h (Table 1). Under both conditions, DCs displayed the expected immature phenotype (29,

Table 1. Membrane marker expression by human dendritic cells

Markers	Culture conditions ¹	
	Normoxia	Hypoxia
HLA-DR	93 ± 3.5	91 ± 2.2
CD1a	56 ± 2.4	46 ± 1.1 ²
CD11c	63 ± 12	61 ± 7.2
CD14	0.6 ± 0.2	1.1 ± 0.6
CD40	0.3 ± 0.1	0.4 ± 0.2
CD123	3.0 ± 0.9	3.3 ± 1.3
CD80	50 ± 4.8	37 ± 2.0 ²
CD83	1.7 ± 0.1	2.0 ± 0.2
CD86	64 ± 3.0	31 ± 2.8 ²
Lineage (CD3, CD16, CD19, CD56)	0.40 ± 0.01	0.50 ± 0.01

¹Dendritic cells (DCs) were generated from monocytes in the presence of interleukin-4 and granulocyte-macrophage colony-stimulating factor under normoxia for 7 days. DCs were then exposed to either normoxia or hypoxia for 24 h and analyzed using a flow cytometer. Data indicate the mean of positive cells for three donors (mean ± SD).

²Statistically significant difference relative to normoxia ($p < 0.05$).

30): CD14, CD123, and CD49 negativity; CD11c positivity; and strong expression of HLA-DR. Cell size, granularity, and morphology did not differ between DCs under both conditions (data not shown); however, DCs cultured under hypoxia showed a significant reduction in CD80 and CD86 expression, both in terms of their mean channel of fluorescence intensity (data not shown) and the percentage of positive cells (Table 1). Interestingly, the expression of CD1a, a protein that presents lipid antigens to T cells (31), was significantly reduced in DCs under hypoxia. Similar results were obtained with DCs cultured for an additional 24 h (48 h exposure; data not shown), indicating a persistent CD1a, CD80, and CD86 down-regulation under hypoxia. These results were consistently obtained using samples from three healthy donors, indicating that hypoxia influenced the expression of co-stimulatory molecules of DCs.

Hypoxia has been previously shown to induce macrophages to control *Leishmania* infection (9–11). To address the question of whether hypoxia affected this functional ability in DCs, uptake and resistance to the parasite were tested (Fig. 1A,B). The infection index of DC cultures under hypoxia and normoxia conditions was examined. As expected, DCs infected with

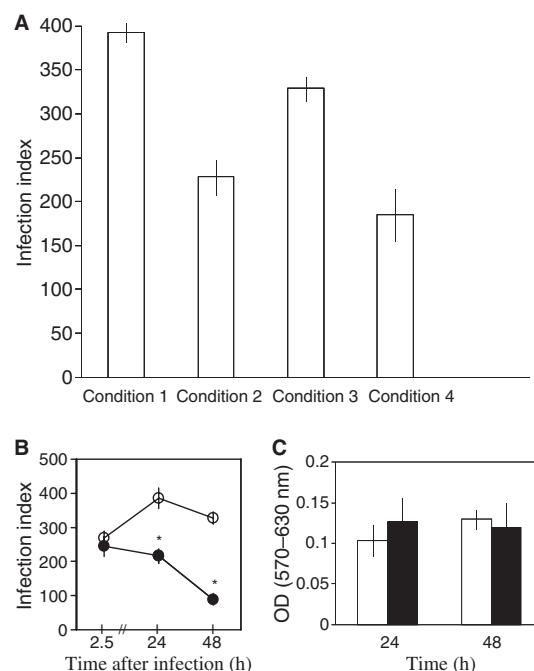


Fig. 1. Effects of hypoxia in human dendritic cells (DCs). (A) DCs were infected with *Leishmania* and cultured under normoxia (condition 1) or hypoxia (condition 2) for 24 h, pre-incubated for 24 h under hypoxia, and infected with *Leishmania* under normoxia for 24 h (condition 3) or pre-incubated for 24 h under hypoxia and infected with *Leishmania* under hypoxia for 24 h (condition 4). The infection index was determined as described in the Materials and methods section. (B) DCs were infected with *Leishmania* for 2.5, 24 and 48 h under normoxic (○) or hypoxic (●) conditions. (C) MTT production by DCs was determined in cell cultures maintained in normoxia (□) or hypoxia (■) for the time indicated. Data represent the mean ± SD of one representative experiment of three or four independently performed experiments with similar results. * $p < 0.05$.

parasites and maintained under normoxia for 24 h showed a high infection index (condition 1; Fig. 1A), whereas DCs exposed to hypoxia at the time of infection (condition 2) showed a significant reduction of 40% in parasitized cells compared with that of DCs exposed to normoxia (condition 1). Likewise, DCs incubated under hypoxia 24 h before infection and then infected under hypoxia (condition 4) exhibited a significant reduction in infection index (approximately 50%). In contrast, DCs only exposed to hypoxia before infection and then cultured and infected under normoxia showed no reduction in the infection index (condition 3) (Fig. 1A). The

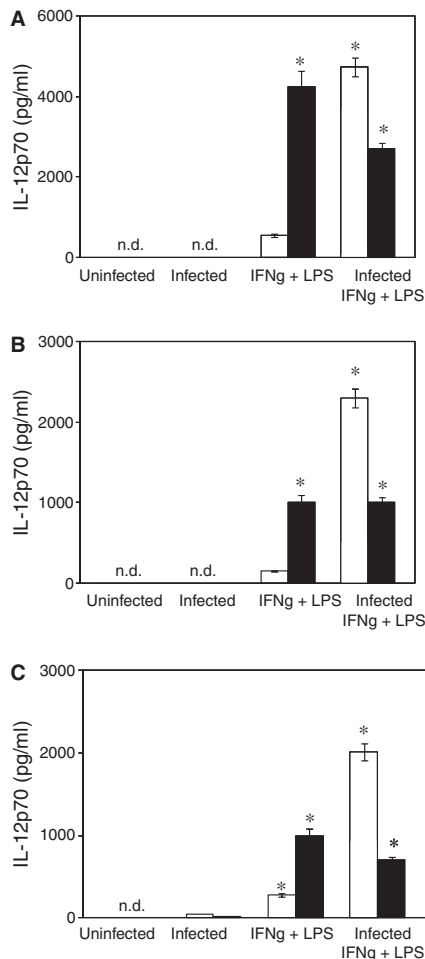


Fig. 2. Effect of hypoxia on interleukin-12 (IL-12) production by human dendritic cells (DCs). DCs cultured under normoxia (□) or hypoxia (■) were uninfected, infected with *Leishmania* (Infected), stimulated with γ -interferon + lipopolysaccharide (IFN γ + LPS) or stimulated with IFN- γ + LPS, and infected with *Leishmania* (infected IFN γ + LPS). After 24 h, the supernatants were tested by ELISA for IL-12p70. Shown are the results obtained with three individual cell donors (A–C). n.d., not detectable. * $p < 0.05$.

results presented in Fig. 1A indicate that the resistance to *Leishmania* induced in DCs by hypoxia is dependent on the combination of hypoxia–normoxia conditions. Next, the hypothesis that DCs under hypoxia show reduced *Leishmania* infection because the uptake of parasites is impaired under hypoxia was tested. To achieve this, the parasites were cultured with DCs for 2.5 h to permit invasion. The DC cultures were then washed to remove

extracellular parasites and immediately counted for infection index or incubated for a period of 24 and 48 h. As shown in Fig. 1B, a similar infection index was obtained immediately after infection (2.5 h), irrespective of whether the DCs were cultured under normoxia or hypoxia. The results presented in Fig. 1B indicate that ingestion of the parasite was identical for DCs cultivated under normoxia or hypoxia. In addition, the results indicate that the resistance to *Leishmania* induced in DCs by hypoxia is dependent on the time that the cells remain in hypoxia (for at least 24 h after infection). *Leishmania* exists in two forms, amastigote and promastigote, and similar results were obtained using cultures of DCs infected with amastigotes DCs (Fig. 1) and cultures of DCs infected with promastigotes (data not shown). It should be noted that DC viability, tested using MTT assay (formazan production by cell mitochondria), was similar at 24 and 48 h after exposure to normoxia and hypoxia (Fig. 1C).

Figure 2 shows the levels of IL-12p70 secretion by DCs from three different donors. Uninfected cells cultured in medium alone under normoxia or hypoxia produced no detectable IL-12p70. Little or no IL-12p70 secretion was observed in DCs infected with *Leishmania* under normoxia or hypoxia. DCs secreted IL-12p70 in response to IFN- γ + LPS in normoxia, which is in agreement with previously published data (28), although this secretion was approximately sixfold greater under hypoxia (Fig. 2). When DCs were stimulated with IFN- γ + LPS and infected with *Leishmania* under normoxia, they produced consistently more IL-12p70 than DCs stimulated with IFN- γ + LPS and infected with *Leishmania* under hypoxia. A similar IL-12p70 production pattern was observed in the supernatants of DC cultures following 48 h of normoxia and hypoxia (data not shown).

DISCUSSION

It is clear from this and previous studies that reduced oxygen tension alters the functional capabilities of DCs (15–24, 32). Our interest concerned the events associated with low oxygen tension in injured and inflamed tissues; thus the study focused on immature DCs that reside in and migrate through diseased tissues (33). In

this work, the results established that although hypoxia does not alter human DC viability, it significantly alters phenotypic characteristics, IL-12 production and response to *Leishmania* infection. Analysis of the results showed that hypoxia without any other stimulus reduced the expression of CD80 and CD86, which are co-stimulatory molecules in T-cell activation, and CD1a, a protein involved in lipid presentation (31, 32). Besides the expression observed for these CD markers, similarity in the cytometric analyses was paralleled by similar morphological appearance after hypoxia exposure. Few works have analyzed the effects of hypoxia in DCs in comparison with the extensive studies published involving macrophages (3, 4, 15–24, 32). Recently, Mancino *et al.* (15) observed lower expression of the markers CD1a, CD40, CD80, CD83, and CD86 in human DCs under hypoxia, with or without LPS stimulation, whereas Jantsch *et al.* (20) and Goth *et al.* (19) reported lower expression of CD80 and CD86 in murine DC under hypoxia alone. Other reports show that hypoxia caused an increase in HLA and CD80 marker expression (22, 23). Despite the different results in these studies, which probably reflect the different protocols used, such as the degree and duration of hypoxia, it can be concluded that modulation in CD markers was determined in DCs under hypoxic conditions. In addition to interfering in the expression of co-stimulatory molecules of DCs, hypoxia targets the pro-inflammatory function of DCs by increasing tumor necrosis factor- α (TNF- α), IL-1, and IFN- γ secretion (15, 16). As shown in this study, DC secretion of IL-12p70, the bioactive IL-12, a cytokine produced in response to inflammatory and microbial mediators (34), was enhanced under hypoxia (Fig. 2). The results of this study are in agreement with those of Murata *et al.* (16), who determined that IL-12 production by murine DCs was increased under hypoxia whether in the presence or absence of IFN- γ and IL-18 stimulation. In contrast with these results, others have reported the inability of hypoxia to induce enhanced IL-12 production in human and murine DCs and even hypoxia-related suppression of IL-12 expression (17, 20, 24). Moreover, one possible explanation for these conflicting results could be the different protocols used, including the degree and duration of

hypoxia, and different IL-12 activation stimuli. Based on the data indicating that hypoxia strongly upregulated pro-inflammatory cytokines TNF- α and IL-1, and the inflammatory chemokine receptor CCR5 and did not suppress endocytosis, a specialized function of DCs, Mancino *et al.* (15) previously proposed that hypoxia promotes a phenotypic adaptation of DCs. This DC phenotype would be characterized by pro-inflammatory antimicrobial activities and could be helpful in the early host response against damage signals (i.e., infection or trauma). In this context, the hypothesis that DCs under hypoxia are able to control the intracellular pathogen *Leishmania* was tested. To the best of our knowledge, no other study has been designed to test this supposition. The current experiments indicate that phagocytic activity (*Leishmania* uptake) is not impaired under hypoxia and extend Mancino's data (15) regarding the endocytic/phagocytic abilities of hypoxic DCs. More interestingly, observations confirmed that hypoxia induced DCs to reduce parasite survival, consequently controlling the infection rate. It is important to point out that similar results have been obtained with macrophages infected with the same *Leishmania* species, *L. amazonensis* (9–11). Interestingly, our group has shown that *L. amazonensis* induces DCs and macrophages under normoxic or hypoxic conditions to express HIF-1 α , which plays a central role in hypoxic expression of a variety of genes (26). Ascertaining whether HIF-1 α induction in DCs is associated with an adaptive mechanism that maintains DCs integrity or a parasite strategy to sustain infection should be the object of further investigation.

Interleukin-12-producing DCs and macrophages are essential for host defense against intracellular pathogens (33). Marked differences in DC response to *Leishmania* species have been reported; in contrast to high level of IL-12 produced in DCs infected with *L. major* and *L. donovani*, *L. amazonensis* infection induces low amounts of IL-12 (14, 35–38). The weak IL-12 production following *L. amazonensis* infection has been associated with a non-healing disease in the most inbred mouse strains and T-cell suppression in patients with a diffuse cutaneous leishmaniasis (14, 38, 39). While evaluating IL-12 production in response to *L. amazonensis* infection with IFN- γ \pm LPS

pre-activated DCs under hypoxia, observation revealed IL-12p70 induction, but at levels approximately half than those produced by DCs cultured under normoxia with the same stimulus and infected with the parasite (Fig. 2). Perhaps more importantly, our data indicate no correlation between IL12-p70 production and reduction in infection in DCs under hypoxia (Fig. 2). Our group is currently investigating the mechanism by which DCs and macrophages are able to control *Leishmania* infection in hypoxia.

In summary, the data corroborate the existing body of literature demonstrating that hypoxia affects DC phenotype and function (15–24, 32). This study demonstrated, for the first time, that hypoxia induced DC resistance to *Leishmania*, but did not impair ingestion of the parasite. Thus, hypoxia modulated co-stimulatory molecules, limiting antigen presentation to T cells, impaired migration in response to chemokines, and enhanced the production of inflammatory cytokines (TNF- α , IL-1, IL-6, and IL-12) and microbicidal activity (15 and results shown in this study). All these data support the notion that a hypoxic microenvironment promotes selective pressure on DCs to assume a ‘pre-mature’ phenotype in injured or inflamed tissues and contribute to the innate immune response.

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