# Functional Alterations in Macrophages After Hypoxia Selection

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**Regions of low oxygen tension are common features of inflamed and infected tissues and provide physiologic selective pressure for the expansion of cells with enhanced hypoxia tolerance. The aim of this study was to investigate whether macrophages resistant to death induced by hypoxia were accompanied by functional alterations. A mouse macrophage cell line (J774 cells) was used to obtain subpopulations of death-resistant macrophages induced by long-term exposure to severe hypoxia (<1% $O_2$). The results indicated that exposing J774 macrophages to periods of severe hypoxia results in the selection of cells with phenotypes associated with the modulation of heat-shock protein 70 kDa (HSP70) expression, tumor necrosis factor-α (TNF-α), and nitric oxide (NO) production and reduced susceptibility to parasite Leishmania infection. Thus, we suggest that hypoxia-selected macrophages may influence the outcome of inflammation and infection.** Exp Biol Med 232:88–95, 2007

**Key words:** hypoxia; selection; macrophages; inflammatory responses; Leishmania

Regions of low oxygen tension (hypoxia) are common features of tumors, wounds, atherosclerotic lesions, and inflamed/infected tissues (1–4). Since cell growth and survival critically rely on adequate oxygen supply, hypoxia provides physiologic selective pressure for the expansion of cells with enhanced hypoxia tolerance (5). Many studies have demonstrated that hypoxia selection of death-resistant tumor cells involves the regulation of p53, a tumor suppressor gene, and defects in the cell death mechanism, such as the upregulation of antiapoptotic proteins (6–11).

Macrophages, cells present in diseased tissues, display functional and phenotypic heterogeneity (12, 13). Moreover, macrophages adapt metabolically and energetically to reduced oxygen tension through the glycolytic processing of glucose (2, 14). Recent studies have shown that in macrophages, hypoxia alters viability, metabolic and phagocytic activities, the release of cytokines, and susceptibility to microorganism infections (15–18). Most importantly, Yun *et al.* (19) demonstrated that subpopulations of macrophages resistant to chronic hypoxia exposure display differential phenotypic characteristics with respect to low-density lipoprotein (LDL) uptake, heat-shock protein 70 kDa (HSP70) expression, and tumor necrosis factor-α (TNF-α) production. The authors suggested that macrophages that are adapted to the hypoxic stress of atherosclerotic lesions may play a critical role in the perpetuation of inflammatory vascular sites (19).

Our laboratory has been interested in the influence of low oxygen tension on macrophage response to parasite stimulus (16, 20, 21). Macrophages treated with a single exposure to mild hypoxia (6% $O_2$) and infected with *Leishmania amazonensis* were able to reduce intracellular parasitism (16, 20). This protozoan is an intramacrophage parasite that causes chronic human diseases ranging from localized to diffuse cutaneous infections (22, 23). The former is the most common form of leishmaniasis and is characterized by a skin ulcer presenting a raised, expanded border of parasite-infected macrophages with an incomplete granulomatous reaction and a necrotic tissue center (22, 24). These lesions self-heal over months or years (22). Diffuse cutaneous leishmaniasis is associated with cutaneous metastases from the initial skin lesion (22, 23). Since chronic infectious diseases, such as leishmaniasis, result in long-term hypoxic tissue or repeated hypoxia stress (21, 25), the occurrence of resistant macrophages by means of hypoxia selection is highly likely. The selection of such cells may contribute to the control or chronicity of leishmaniasis and interfere with the treatment outcome. In this study, a mouse macrophage cell line (J774 cells) was used to obtain subpopulations of death-resistant macrophages induced by long-term exposure to severe hypoxia

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(<1% $O_2$). These hypoxia-selected macrophages were evaluated with regard to HSP70 expression, TNF-α secretion, nitric oxide (NO) production, and parasiticidal activity.

## Materials and Methods

**Reagents.** All reagents were purchased from Sigma Chemical Co. (St. Louis, MO), except recombinant murine TNF-α (BD Biosciences, San Diego, CA); anti–hypoxic-induced transcription factor 1 α (anti–HIF-1α) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA); fetal calf serum (FCS) (Cultilab, Campinas, Brazil); cell culture plates, flasks, and glass coverslips (Nalge Nunc International, Roskilde, Denmark); glycerol; 2-mercaptoethanol; ethylenediaminetetraacetic acid; pepstatin; phenylmethylsulfonyl fluoride; and nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ).

**Cell Cultures and Parasite.** RPMI 1640 medium was reconstituted according to the manufacturer's instructions and was supplemented with 25 μg/ml gentamicin, 2 m*M* L-glutamine, 100 m*M* HEPES, and 10% FCS. The murine macrophage cell line J774 obtained from the American Type Culture Collection (Manassas, VA) and the murine fibroblast cell line L929 obtained from Banco de Células do Rio de Janeiro (Rio de Janeiro, Brazil) were maintained in RPMI 1640 medium as previously described (26, 27). *Leishmania amazonensis* (MHOM/BR/73/M2269) amastigotes were isolated from active skin lesions of BALB/c mice as previously described (28). The parasites were suspended in RPMI 1640 medium and used immediately after isolation.

**Hypoxic Selection of J774 Macrophages.** J774 macrophages cultured in RPMI 1640 medium were placed into in a gas-tight modular chamber (Billups-Rothenberg, Del Mar, CA). The chamber was gassed for at least 15 mins at a flow rate of 2 liters per minute 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 48-hr intervals using a Fyrite apparatus (Bacharach Inc., Pittsburgh, PA). 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 oxygen tension in the culture medium under hypoxia conditions was 7 mm Hg, and it was 150 mm Hg under normoxia conditions ($O_2$ analyzer YSI/53; Yellow Springs Instruments Inc., Yellow Springs, OH). The pH of the medium was 7.4 and did not change significantly during the course of the experiments. After 48 hrs of hypoxia exposure, detached J774 macrophages were removed with the RPMI 1640 medium. The remaining attached cells were then cultured in fresh medium and exposed again to hypoxia for 48 hrs. This procedure was repeated five times with the attached cells that survived each round of hypoxia treatment. After five hypoxic cycles, selected macrophages were immediately used for experiments (Jsel) and also were cultured under normoxic conditions for 15 days (Jsel-15). Cell viability was determined by trypan blue exclusion, ability of adherence, and cell growth (16).

**Macrophage Activation and Infection with *L. amazonensis*.** For the macrophage activation experiments, the cells were treated with 20 ng/ml mouse interferon γ (IFN-γ) and 10 ng/ml lipopolysaccharide (LPS) from *Escherichia coli* for 24 hrs (26). J774 macrophages were infected by adding to the cell cultures a suspension of living *L. amazonensis* amastigotes in RPMI 1640 medium with a 3-fold excess of parasites for 24 hrs. After the infection period, the cultures were washed to remove extracellular parasites. For the evaluation of the infection index (percentage of infected macrophages × number of amastigotes per macrophage), cells on coverslips were stained with Giemsa and examined microscopically at ×1000 magnification (28, 29).

**TNF-α Bioassay.** The culture 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 (27, 30). The sensitivity of the L929 cell line was determined by using recombinant murine TNF-α. After 24 hrs of incubation, the plates were fixed in 20% methanol and stained with 0.5% crystal violet before cytotoxicity quantitation with an automated micro-ELISA reader (Labsystem Multiskan MS, Helsinki, Finland). The cytotoxicity effects by supernatants containing TNF-α activity were calculating using the following formula: cytotoxicity (%) = $[OD_{540}$ control − $OD_{540}$ test$]/OD_{540}$ control × 100, where control and test represent absorption of L929 plus medium and L929 plus supernatants, respectively (30).

**Nitrite Assay.** A colorimetric assay measured nitrite content in the supernatant of macrophage cultures (31). Supernatants were incubated with Griess reagent, and the absorbance at 540 nm was determined. Nitrite quantities were calculated from a standard curve with serial dilutions of sodium nitrite in RPMI 1640 medium. Each sample was assayed in triplicate, and the results are mean values ± SD.

**Immunoblotting Analyses.** After different treatments, the macrophages were scraped from the culture flasks, checked for viability, and then rinsed twice with phosphate-buffered saline. Lysis buffer (62.5 m*M* Tris-HCl, pH 6.8; 69 m*M* sodium dodecyl sulfate [SDS]; 10% glycerol; 2% 2-mercaptoethanol; 34 m*M* ethylenediaminetetraacetic acid; 2 μg/ml pepstatin; and 1 m*M* phenylmethylsulfonyl fluoride) was added to the cell pellets. Proteins were denatured at 95°C for 3 mins, electrophoresed on a 10% SDS–polyacrylamide gel system (Thermo EC, Holbrook, NY) and transferred to nitrocellulose membranes. After blotting, the membranes were incubated with mouse monoclonal anti-HSP70, anti–inducible nitric oxide synthase (anti-iNOS), or rabbit polyclonal anti–HIF-1α antibodies. The secondary antibody consisted of peroxidase-

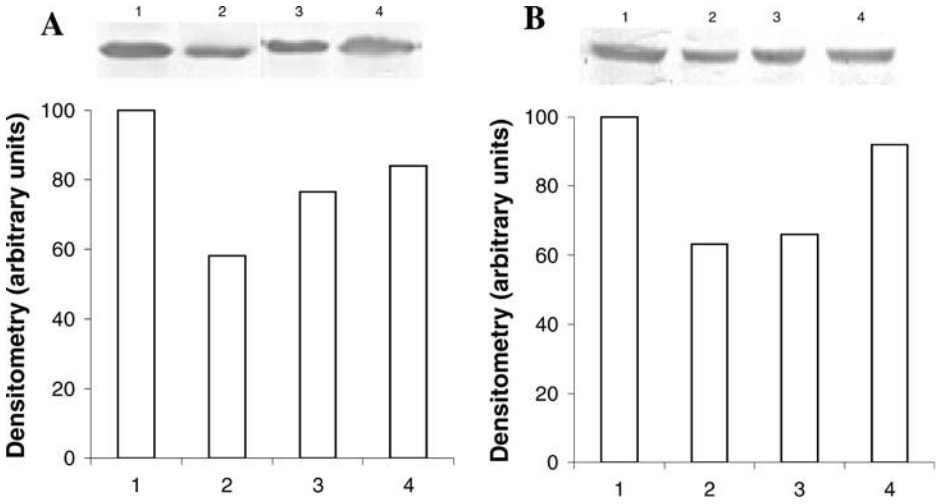


**Figure 1.** Western blot analyses of HSP70 in J774 and hypoxia-selected macrophages. (A) J774 cells cultured in normoxia for 24 hrs (lane 1), J774 cells cultured in hypoxia for 24 hrs (lane 2), Jsel cells cultured in normoxia for 24 hrs (lane 3), Jsel cells cultured in hypoxia for 24 hrs (lane 4). Graph shows the results of Western blots analyzed by densitometry. (B) J774 cells cultured in normoxia for 24 hrs (lane 1), J774 cells cultured in hypoxia for 24 hrs (lane 2), Jsel-15 cells cultured in normoxia for 24 hrs (lane 3), Jsel-15 cells cultured in hypoxia for 24 hrs (lane 4). Graph shows the results of Western blots analyzed by densitometry.

conjugated rabbit anti-mouse IgG or goat anti-rabbit IgG; development was performed with 3,3-diaminobenzidine. Immunoreaction images were scanned, and the densitometric value of each band was determined using Image Master Total Lab version 1 software (Amersham Pharmacia Biotech; Ref. 20).

**Statistical Evaluation.** The results are expressed as mean ± SD. Data obtained from the experiments with J774, Jsel, and Jsel-15 macrophages were analyzed statistically by the Student *t*-test, with a significance level set at $P < 0.05$ or $P < 0.01$.

## Results

**Selection of Hypoxia-Induced Death-Resistant Macrophages.** Before any experiments of hypoxic selection, the induction of biologically relevant hypoxia in our cell system was confirmed by immunoblotting analyses of HIF-1α expression in J774 macrophages exposed to severe hypoxia (<1% $O_2$) for 24 hrs. In contrast, HIF-1α could not be detected in J774 macrophages maintained in normoxia (data not shown). Our strategy was to subject J774 macrophages to hypoxia to kill vulnerable cells and select resistant macrophages. To this end, macrophages were exposed to hypoxia for 48 hrs, and after this first hypoxic cycle the detached cells were removed and the remaining attached macrophages were again exposed to hypoxia. This procedure was repeated five times with the attached cells that survived each round of hypoxia exposure. The cell viability during the first hypoxic cycle was about 40%. The macrophage viability rate increased to about 80% and 90% after three and five sequential hypoxic cycles, respectively, demonstrating that the selected macrophages became resistant to death induced by hypoxia. No differences in morphology, proliferation, and adhering ability were observed between J774 macrophages and hypoxia-selected macrophages (Jsel; data not shown).

**Expression of HSP70 by Hypoxia-Selected Macrophages.** HSP70 is a heat-shock protein expressed constitutively in macrophages, and previous studies indicated a reduction in HSP70 expression level in cells after a single period (24 hrs) of mild hypoxia (≥6% $O_2$; Refs. 20, 32, 33). To see whether similar changes in HSP70 expression were detectable in hypoxia-selected macrophages, Western blots of HSP70 were performed. As shown in Figure 1, HSP70 was constitutively expressed in J774 macrophages in normoxia, and a 40% reduction in HSP70 was observed after exposure to hypoxia (<1% $O_2$) (Fig. 1A, lanes 1 and 2). Jsel macrophages maintained in normoxia for 24 hrs expressed HSP70 at lower levels than J774 cells cultivated in normoxia (Fig. 1A, lanes 1 and 3). In contrast to J774 macrophages, the level of HSP70 in Jsel cells was not reduced after 24 hrs of hypoxia (Fig. 1, lanes 3 and 4). Notably, the HSP70 expression pattern of Jsel cells was maintained in this macrophage subpopulation, even after 15 days of cell culture under normoxia conditions (Jsel-15); that is, they showed reduced HSP70 expression compared with J774 macrophages cultivated in normoxia (Fig. 1B, lanes 1 and 3) and increased HSP70 expression when cultured for 24 hrs in hypoxia (Fig. 1B, lanes 3 and 4). These data indicate that hypoxia selection of a distinct HSP70-related phenotype was not a transient phenomenon but was retained in macrophages for at least 2 weeks under ambient $O_2$ conditions.

**Production of TNF-α and NO by Hypoxia-Selected Macrophages.** We further examined whether the selection of hypoxia-resistant macrophages affected the ability to produce two biomolecules, TNF-α and NO, involved in inflammatory responses (34, 35). J774, Jsel, and

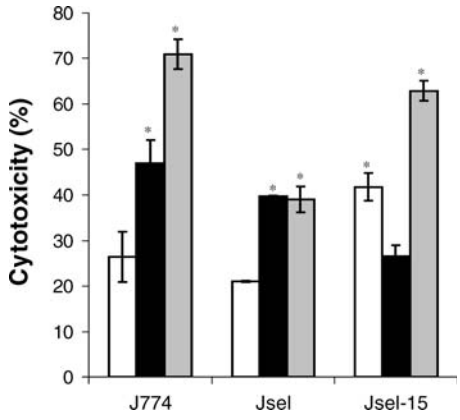


**Figure 2.** TNF-α production of J774 and hypoxia-selected macrophages. TNF-α bioactivity from culture supernatants of J774, Jsel, and Jsel-15 cells exposed to normoxia (white bars) or hypoxia (black bars), or stimulated with IFN-γ + LPS in normoxia (gray bars) for 24 hrs, were determined in the L929 cytotoxicity assay. The results presented were obtained in triplicate L929 cultures ± SD of one representative experiment. *$P < 0.01$ compared with culture supernatants of J774 cells in normoxia.

Jsel-15 macrophages were cultured under normoxia or hypoxia conditions or were stimulated with IFN-γ plus LPS, a well-defined proinflammatory activator system (35, 36), and 24 hrs later supernatants were collected and assayed for TNF-α bioactivity. As shown in Figure 2, a significant difference in TNF-α release between J774, Jsel, and Jsel-15 release was observed. The highest TNF-α bioactivity was observed in supernatant cultures of IFN-γ + LPS–exposed J774 cells (Fig. 2). The Jsel macrophage cultures presented a significantly lower TNF-α release than J774 and Jsel-15 cell cultures when treated with the same stimulus (IFN-γ + LPS). When the three macrophage populations were exposed to 24 hrs of hypoxia, TNF-α bioactivity was enhanced in J774 and Jsel supernatants (Fig. 2). Notably, hypoxia reduced TNF-α bioactivity in Jsel-15 supernatant cultures (Fig. 2).

To examine whether the selection of hypoxia-resistant macrophages also affected their ability to produce NO, the macrophages were treated with IFN-γ + LPS and assayed for nitrite accumulation in the supernatants (Fig. 3A). As previously reported, high levels of nitrite were induced in J774 macrophage cultures stimulated with IFN-γ + LPS in normoxic conditions (26). In contrast to J774 cells, Jsel and Jsel-15 macrophages produced low levels of nitrite in response to the activators (about 30% of J774 macrophage nitrite accumulation; Fig. 3A). It should be noted that the three subpopulations of macrophages (J774, Jsel, and Jsel-15 cells) produced minimum quantities of nitrite under hypoxic condition (data not shown). The question of whether increased nitrite production during activation with IFN-γ + LPS in normoxia is related to increased iNOS expression in macrophages was addressed by measuring the levels of iNOS protein in cell lysates. As shown in Figure 4B, J774 cells treated with IFN-γ + LPS showed a major increase in iNOS protein levels (Fig. 3B, lane 1). iNOS also increased after IFN-γ + LPS stimulation in Jsel and Jsel-15 macrophages; however, the iNOS protein level was about 40% lower than in J774 cells (Fig. 3B and C). There was no detectable iNOS protein in nonstimulated macrophages cultured under normoxia or hypoxia (data not shown). Thus, a differential response of hypoxia-selected macrophages to inflammatory stimulus was demonstrated by the differences

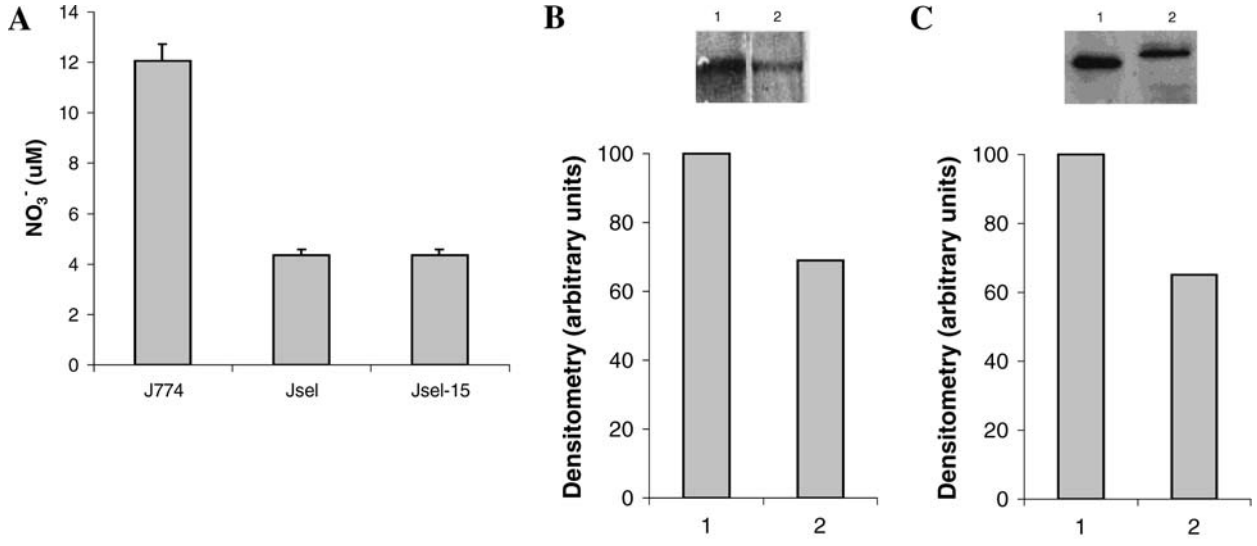


**Figure 3.** NO production of J774 and hypoxia-selected macrophages. (A) J774, Jsel, and Jsel-15 macrophages were stimulated with IFN-γ + LPS in normoxia for 24 hrs. Nitrite concentrations were determined in the supernatants from cultures by the Griess reaction. The results represent the mean ± SD of one representative experiment. *$P < 0.05$ compared with culture supernatants of J774 cells stimulated with IFN-γ + LPS. (B) Western blot analyses of iNOS in J774 (lane 1) and Jsel (lane 2). Graph shows the results of Western blots analyzed by densitometry. (C) Western blot analyses of iNOS in J774 (lane 1) and Jsel-15 (lane 2). Graph shows the results of Western blots analyzed by densitometry.

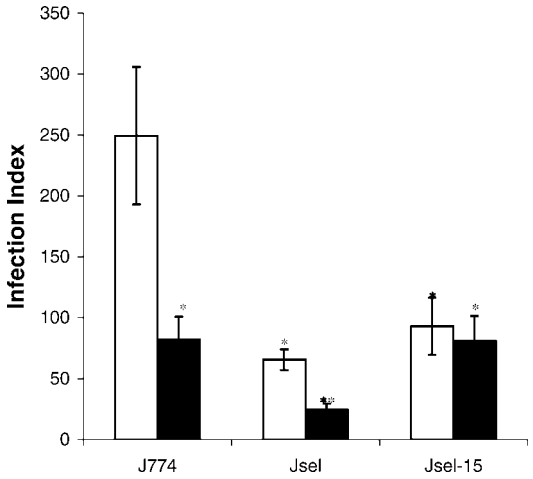


**Figure 4.** Leishmania infection in J774 and hypoxia-selected macrophages. J774, Jsel, and Jsel-15 macrophages were infected with Leishmania amastigotes and cultured in normoxia (white bars) or hypoxia (black bars). After 24 hrs the IF was determined as described in Materials and Methods. The results represent the mean ± SD of one representative experiment. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with J774 macrophages infected with Leishmania amastigotes and cultured in normoxia.

in the levels of TNF and NO production observed between J774, Jsel, and Jsel-15 cells.

**Susceptibility of Hypoxia-Selected Macrophages for an Intracellular Pathogen.** To address the question of whether the selection of hypoxia-resistant macrophages affected functional ability, the susceptibility of the cells to an intracellular pathogen, Leishmania, was analyzed. Macrophages are the host cells for Leishmania, where these parasites are able to survive and replicate (37). As expected, J774 macrophages cultured with Leishmania and maintained under normoxia for 24 hrs showed a high infection index (IF) (Fig. 4). However, in J774 cell cultures subjected to hypoxia, a reduction in IF occurred (Fig. 4). Notably, Jsel macrophages exposed to normoxia during the period of infection showed a significant reduction in infection compared with J774 cells (Jsel IF: 65; J774 IF: 250; Fig. 4). A more pronounced reduction in IF was observed when Jsel macrophages were infected under hypoxia (IF: 24, Fig. 4). The ability of hypoxia-selected macrophages to resist Leishmania infection also was maintained in Jsel-15 cells. IF was significantly suppressed in Jsel-15 cells exposed to normoxia and hypoxia during the period of infection (Fig. 4). These results indicate that hypoxia-selected macrophages are more resistant to an intracellular pathogen infection.

## Discussion

It is clear from this and previous studies (6–11, 19) that hypoxic-rendered cell populations show death resistance to microenvironments with low oxygen pressure. Altered characteristics in tumor cells subjected to selective pressure, such as hypoxia, have been reported, and the selected cells generally are examined by growth characteristic, resistance to apoptotic stimulus, and response to DNA-damaging drugs (9–11). The aim of the present study was to investigate whether death-resistant macrophages induced by hypoxia selection were accompanied by functional changes. The J774 macrophage cell line was used to obtain two subpopulations of death-resistant macrophages induced by long-term exposure to severe hypoxia ($<1\%$ $O_2$): Jsel, assayed immediately after five cycles of hypoxia; and Jsel-15, derived from Jsel macrophages and cultured for 15 days in normoxia before use. We found that HSP70, a protein that has been correlated with cell resistance to microenvironment stress (38), was differentially expressed in J774, Jsel, and Jsel-15 macrophages. The expression level of HSP70 was reduced in J774 macrophages exposed to hypoxia, whereas the HSP70 level was not altered in Jsel macrophages. Jsel-15 cells express low levels of HSP70 in normoxia and high levels of HSP70 in hypoxia. Yun *et al.* (19) also noted that the RAW macrophage cell line exposed to hypoxia (2% $O_2$) expressed low levels of HSP70, and selected macrophages maintained the same HSP70 levels under hypoxia or normoxia. The present results agree with their findings and indicate that low levels of HSP70 in macrophages coincide with poor resistance to severe hypoxia. Since the present work examined a subpopulation of macrophages cultured for 15 days in normoxia before the tests (Jsel-15), which showed enhanced levels of HSP70 after hypoxic stimulus, it is possible to confirm that HSP70 is a marker for adaptation to severe hypoxic stress.

The present study also demonstrated macrophage variability in the pattern of TNF-α and NO production. These two biomolecules are involved in macrophage cytotoxic and inflammatory responses (34, 35). The results demonstrated maximum TNF-α bioactivity in J774 macrophage cultures after IFN-γ + LPS stimulation, an intermediate TNF-α production by cells cultured under hypoxia, and low TNF-α production by nonstimulated cells. In Jsel macrophages, an intermediate TNF-α production was observed in cultures stimulated with IFN-γ + LPS or maintained in hypoxia. In contrast, Jsel-15 cells produced maximum TNF-α levels when stimulated with IFN-γ + LPS, but they did not respond to a hypoxic microenvironment. These results support previous observations that hypoxia (1%–9% $O_2$) induces TNF-α release in unstimulated or endotoxin-stimulated human mononuclear cells and macrophage cell lines (27, 39–42). However, Yun *et al.* (19) reported a suppressive effect of hypoxia (2% $O_2$) on the murine macrophage cell line RAW 264.7, although hypoxia-selected RAW macrophages secreted TNF-α when exposed to hypoxia (19). Thus, despite the different results achieved in experiments with these two parental macrophages (J774 and RAW cells), which likely reflect differences in cell line type, the present results with hypoxia-selected macrophages are in agreement with those by Yun *et al.* (19), indicating a distinct phenotype for TNF-α production in hypoxia-selected macrophages. The current

experiments extend the studies by Yun *et al*. (19) and reinforce the hypothesis that a distinct functional phenotype is originated after hypoxia selection. Hypoxia-selected macrophages also differ from J774 macrophages with respect to NO production. The present results indicated that under normoxic conditions, IFN-$\gamma$ + LPS–activated Jsel and Jsel-15 macrophages produce less nitrite and iNOS protein than IFN-$\gamma$ + LPS–activated J774 macrophages. Although no reports on the effect of hypoxia selection on NO production by selected cells are available for comparison with these results, a number of studies have reported that nitrite accumulation and iNOS protein in macrophages and myocytes are decreased in proportion to oxygen tension (42–46). The absence of oxygen as a substrate (43, 45), an increase in cysteine protease calpain expression contributing to iNOS degradation (47), and a lack of tetrahydrobiopterin cofactor and auxiliary proteins (43, 48), resulting in the displacement and instability of the iNOS structure (49), are used to explain low NO production in cells exposed to hypoxia. Whether transcriptional or posttranscriptional events are involved in decreased NO and iNOS production in hypoxia-selected macrophages remains to be established.

To evaluate whether another functional marker was altered in hypoxia-selected macrophages, the ability to maintain or endure infection by an intracellular parasite, Leishmania, was analyzed in J774, Jsel, and Jsel-15 cells. Macrophages are hosts for Leishmania and are the major site for parasite replication (37). The results demonstrated that hypoxia caused a significant decrease in the J774 macrophage IF by Leishmania. More interestingly, we found that hypoxia-selected macrophages are resistant to parasites even under normoxic conditions, indicating that hypoxia selection confers a Leishmania-resistant phenotype to macrophages. To our knowledge no previous studies have investigated the effect of cell hypoxia selection with regard to microorganism infection. Yun *et al*. (19) demonstrated that hypoxia-selected RAW macrophages take up a minimum level of acetylated LDL in normoxia (19). Although direct comparison with these results is not possible, collectively they indicate deficient phagocytosis in hypoxia-selected macrophages, which could be the effect of alterations in cytoskeleton protein rearrangement, changes in ligands and receptors, and membrane trafficking (19, 49, 50). In support of this interpretation, a number of studies have reported the distinct changes in gene and protein expression that occur in macrophages and other cell types when they experience *in vitro* hypoxia (49, 51–53). These include the modulation of molecules required for metabolism, transcriptional factors, and membrane-bound proteins (e.g., CD80) and proteins associated with signaling cascade (17, 41, 49, 51–53).

In summary, this study shows that exposing macrophages to periods of severe hypoxia results in the selection of cells with phenotypes associated with the modulation of HSP70 expression, TNF-$\alpha$ and NO production, and Leishmania susceptibility. This variability in the pattern of HSP70 expression, TNF-$\alpha$ and NO production, and parasite susceptibility in hypoxia-selected and nonselected J774 macrophage subpopulations suggest that macrophages can progressively change their phenotype in response to progressive changes in microenviromental signals. Many questions remain concerning the complex molecular mechanism that alters macrophage phenotype after hypoxic selection. HIF-1 is a heterodimer consisting of an alpha subunit, the oxygen-responsive component, and beta subunit constitutively expressed in most cells (55). HIF-1$\alpha$ induced under hypoxic conditions regulates the expression of several genes, including glycolytic enzymes and cytokines (55). It has recently been shown that during functional maturation from monocytes to macrophages HIF-1 activity increases, and cells acquire the ability to respond to hypoxia more robustly (56). Since HIF-1$\alpha$ expression could be detected in J774 macrophages under hypoxic conditions as chosen for our experiments, we can speculate that HIF-1$\alpha$ is involved in the phenotype and functional changes observed in hypoxia-selected macrophages.

Considering the presence of hypoxia in inflammatory and infectious lesions from initiation to resolution, it is likely that injured tissues would contain hypoxia-selected macrophages with the attenuated inflammatory phenotype, which would facilitate tissue regeneration and homeostasis. Therefore, knowledge regarding the functional state and plasticity of macrophages (54) challenged by the tissue microenvironment could be of paramount importance for drug and vaccine design.

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