UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DE BIOLOGIA

ADRIANA DEGROSSOLI

"ESTUDOS DE ALTERAÇÕES FUNCIONAIS DE MACRÓFAGOS SUBMETIDOS A HIPÓXIA NO MODELO *IN VITRO* DA LEISHMANIOSE"

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Genética e Biologia Molecular, na área de Imunologia.

Orientadora: Profa. Dra. Selma Giorgio

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BANCA EXAMINADORA

Profa. Dra. Selma Giorgio (Orientadora)

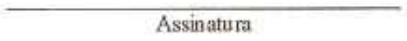
Prof. Dr. Phileno Pinge Filho

Profa. Dra. Anita Hilda Strauss Takahashi

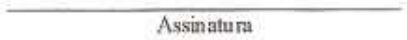
Profa. Dra. Maria Isabel Nogueira Cano

Profa. Dra. Liana Maria Cardoso Verinaud

Profa. Dra. Silmara Marques Allegretti



Prof. Dr. Marcelo Brocchi



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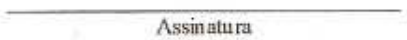

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"A ignorância afirma ou nega veementemente. A ciência duvida."

(Voltaire)

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RESUMO

Diversas patologias provocam mudanças na pressão parcial de oxigênio, tornando o microambiente tecidual hipóxico. O interesse em analisar as alterações fenotípicas de células em hipóxia deve-se a necessidade de entender os mecanismos patológicos e a resistência aos tratamentos e ao desenvolvimento de terapias celulares. Células como os macrófagos adaptam-se a hipóxia modificando o metabolismo e a produção de citocinas. As lesões causadas pelo parasita intracelular *Leishmania amazonensis* são hipóxicas e o cultivo de macrófagos (células hospedeiras da *Leishmania*) em hipóxia induz redução da infecção com o parasita e modula a expressão de proteínas do choque térmico, indicando alterações funcionais e estruturais em ambiente hipóxico. Neste trabalho avaliamos os mecanismos responsáveis pela resistência destas células ao parasita em hipóxia e as modificações dos macrófagos causadas por este microambiente. Macrófagos cultivados em hipóxia não apresentam alterações na produção de óxido nítrico (NO) e na expressão da sua enzima produtora, óxido nítrico sintase (iNOS). Além disso, macrófagos *knockout* para enzima iNOS, que não produzem NO, são capazes de reduzir a infecção por *L. amazonensis* semelhante a macrófagos selvagens, o que sugere que o efeito leishmanicida da hipóxia não se deve ao NO. A liberação das citocinas TNF-α, IL-6, IL-12 e IL-10 pelos macrófagos é alterada quando estes são cultivados em microambiente hipóxico. A produção destas moléculas pelos macrófagos infectados com *L. amazonensis* é semelhante em hipóxia e normóxia, indicando que estas citocinas não participam do efeito leishmanicida. O metabolismo energético dos macrófagos infectados, avaliado pela produção de ATP, não é modificado pela hipóxia, indicando que este fator não está envolvido na morte do parasita em macrófagos cultivados em hipóxia. Embora macrófagos fagocitem menos partículas inertes em hipóxia do que macrófagos em normóxia, a fagocitose do parasita vivo não é alterada pela hipóxia, sugerindo que o processo fagocítico também não está relacionado à diminuição da

infecção pela hipóxia. Macrófagos cultivados em hipóxia aumentam a produção de ROS em relação a normóxia, mas a produção de ROS por macrófagos infectados com *L. amazonensis* em hipóxia não é alterada. Mas a inibição do efeito leishmanicida pelos antioxidantes *N*-acetilcisteína e Ebselen sugere que ROS tem papel na resistência dos macrófagos a *L. amazonensis* em hipóxia. A expressão de duas isoformas do fator transcricional hypoxia-inducible factor (HIF) (HIF-1alfa e 2alfa) é induzida em macrófagos cultivados em ambiente hipóxico e, interessantemente, em macrófagos infectados com *L. amazonensis*, mesmo quando estes são cultivados em normóxia. A inibição do HIF-1alfa pelo cloreto de cobalto impediu a sobrevivência do parasita dentro do macrófago, indicando que este fator é importante na manutenção do macrófago como célula hospedeira da *L. amazonensis*. Nossos resultados também demonstraram que a hipóxia alterou a capacidade proliferativa dos linfócitos T e o processamento e apresentação de antígeno de *L. amazonensis* pelos macrófagos. Assim, concluímos que a hipóxia induz alterações funcionais e estruturais nos macrófagos e que ROS são importantes para o efeito leishmanicida da hipóxia.

Palavras-chave: *Leishmania*, macrófagos, hipóxia.

ABSTRACT

Regions of low oxygen tension (hypoxia) are common features of inflamed/infected tissues. The analyses of cell phenotypic alterations by hypoxia are helpful for understanding of the pathological mechanisms and treatment resistance and for the development of cellular therapies. Macrophages, cells involved in the clearance of microorganisms from infected tissues, are influenced by oxygen tension changing metabolism and cytokines production. Lesions caused by intracellular protozoan *Leishmania amazonensis* are hypoxic and macrophages (host cells for *Leishmania*) cultured in hypoxia are resistant to the infection and change heat shock proteins expression, suggesting functional and structural alterations of these cells in hypoxic microenvironment. In the present work we evaluated mechanisms involved in the macrophage resistance to the parasite as well as macrophages phenotypic alterations in hypoxia. Macrophages cultured in hypoxia did not show alterations in nitric oxide (NO) production and oxide nitric synthase (iNOS) enzyme expression. Furthermore, iNOS knockout macrophages lacking NO production are also able to reduce *L. amazonensis* infection as wild type macrophages, what suggests the leishmanicidal effect of hypoxia is not related to NO. The cytokines TNF-α, IL-6, IL-12 e IL-10 release is altered when macrophages are cultured in hypoxia. However, the production of these cytokines by *L. amazonensis*-infected macrophages in hypoxia is similar to normoxia, indicating these cytokines do not participate of the leishmanicidal effect of hypoxia. The energetic metabolism of infected macrophages, evaluated through ATP production, is not modified by hypoxia, suggesting this factor is not responsible for parasite death by macrophages cultured in hypoxia. Although the uptake of inert particles by macrophages in hypoxia is lower than in normoxia, living *L. amazonensis* phagocytosis by macrophages is not altered by hypoxia. This result suggests that phagocytic process is also not related to low infection in hypoxia. Cultured macrophages in hypoxia have shown higher ROS production than in normoxia, but the

ROS production by *L. amazonensis*-infected macrophages is not altered by hypoxia. Indeed, the inhibition of leishmanicidal effect of hypoxia by antioxidants *N*-acetylcystein and Ebselen suggests ROS play a role in the macrophage resistance to *L. amazonensis* in hypoxia. The expression of two isoforms of the transcriptional factor hypoxia-inducible factor (HIF) (HIF-1alpha e 2alpha) is induced in macrophages cultured in hypoxia and, interestingly, in *L. amazonensis*-infected macrophages in normoxia and hypoxia. The inhibition of HIF-1alpha by cadmium chloride impaired survival of intracellular parasite, suggesting this factor is important for macrophages as host cell for *L. amazonensis*. Our results also demonstrated hypoxia altered the proliferative capacity of T lymphocytes and the *L. amazonensis* antigen presentation by macrophages. We conclude hypoxia induces macrophages functional and structural alterations and ROS are important for the leishmanicidal effect of hypoxia.

Keywords: *Leishmania*, macrophages, hypoxia.

SUMÁRIO

1. INTRODUÇÃO

1.1. Relevância fisiopatológica da hipóxia

Hipóxia é um estado de reduzida pressão parcial de oxigênio (pO_2) abaixo de um limiar crítico, que restringe o funcionamento de órgãos, tecidos ou células (KOH; SPIVAK-KROIZMAN; POWIS, 2008; HÖCKEL; VAUPEL, 2001). Os níveis normais de pO_2 variam de 150 mmHg nas vias superiores a aproximadamente 5 mmHg na retina, enquanto uma pO_2 menor que 40 mmHg no sangue arterial constitui hipóxia (KOH; SPIVAK-KROIZMAN; POWIS, 2008). A hipóxia pode ser causada pela redução do suprimento de oxigênio ou pela isquemia localizada causada pela interrupção do fluxo sanguíneo de uma dada área (KOH; SPIVAK-KROIZMAN; POWIS, 2008). A formação de microambientes hipóxicos ocorre em neoplasias, arteriosclerose, fraturas ósseas, artrites e reações inflamatórias em geral (BROWN, 2002; KAUFMAN et al., 2004; LEWIS et al., 1999). Entretanto, a hipóxia também tem um importante e benéfico papel na fisiologia dos mamíferos, constituindo um estímulo fisiológico que ocorre em resposta ao crescimento tecidual durante o desenvolvimento normal e a embriogênese (KOH; SPIVAK-KROIZMAN; POWIS, 2008; SEMENZA, 2009;).

O interesse na análise do fenótipo celular e nas modificações teciduais sofridas em microambiente hipóxico deve-se a necessidade de compreender os mecanismos patológicos e a resistência a diversos tratamentos (GRIFFITHS et al., 2000). Estes estudos são relevantes, também, para o desenvolvimento de técnicas de terapia celular. Os macrófagos são utilizados com freqüência como "veículos" nestas terapias devido à capacidade migratória natural de fagócitos. Algumas dificuldades observadas nestas terapias são atribuídas às modificações que estas células sofrem nos microambientes hipóxicos teciduais lesados (BROWN, 2002; BURKE et

al., 2002; CROWTHER et al., 2001; GRIFFITHS et al., 2000). A avaliação das adaptações celulares ao ambiente hipóxico pode contribuir para a otimização destes tratamentos.

Os dois modelos biológicos mais utilizados nos estudos de hipóxia são tumores e indução de úlceras (revisto por FELDMEIER et al., 2003). Os tumores de mama, por exemplo, apresentam pO_2 de aproximadamente 25 mmHg, enquanto a média encontrada em tecido normal é 65 mmHg; em tumores de pele a pO_2 é 10 mmHg enquanto a pO_2 do tecido subcutâneo humano é 40-60 mmHg (revisto por BROWN, 2002). Em neoplasias, a hipóxia é resultado de dois processos distintos: a perfusão deficiente, que dificulta a difusão do oxigênio através do tecido tumoral, e a proliferação celular descontrolada, que demanda níveis elevados de energia e consumo de oxigênio (BROWN, 2002; FELDMEIER et al., 2003). Experimentos demonstraram que hipóxia induz morte celular por apoptose em linhagem de linfoma T, em células de adenocarcinoma e em fibroblastos SV-40 transformados e sem mitocôndrias (revisto em LEWIS et al., 1999). Entretanto, também foram observadas seleção e resistência celular a hipóxia. Graeber et al. (1996) demonstraram que em ambiente hipóxico há seleção de células tumorais resistentes a apoptose com expressão deficiente de p53.

O outro modelo biológico usado nos estudos dos efeitos da hipóxia é o da indução de ferimentos (úlceras) através de incisões ou implantação de corpo estranho em tecidos dérmicos e/ou cutâneos de roedores ("wound healing") (FELDMEIER et al., 2003; LEIBOVICH; ROSS, 1975; WONG; HOLLINGER; PINERO, 1996). As medidas de tensão de oxigênio em tecido dérmico lesado experimentalmente revelam um padrão temporal e espacial da pO_2 tecidual. Após 1-2 dias de incisão em tecido dérmico de hamster, a pO_2 é de 20-30 mmHg e cai para 5-7 mmHg após cinco dias; o aumento gradual da pO_2 é observado na fase de cicatrização da lesão (HAROON et al., 2000; LEIBOVICH; ROSS, 1975). As baixas tensões de oxigênio das áreas lesadas ocorrem devido à perfusão deficiente causada pelo dano vascular e a intensa atividade

metabólica de células migratórias. Os macrófagos parecem desempenhar papel importante no processo, pois as depleções de monócitos circulantes e macrófagos teciduais resultaram em aumento das áreas necróticas e na redução acentuada do número de fibroblastos e fibrose (LEIBOVICH; ROSS, 1975). A hipóxia está envolvida na indução de diversas citocinas produzidas por células isoladas destas lesões, tais como interleucina-6 (IL-6), fator de necrose tumoral (TNF) e vascular endothelial growth factor (VEGF, um conjunto de proteínas com propriedades mitogênicas para células endoteliais e envolvidas na neovascularização) (CROWTHER et al., 2001; LEWIS et al., 1999). O ambiente hipóxico também induz a expressão de enzimas do metabolismo da glicose, assegurando sobrevivência e funcionalidade celular nas áreas de lesões. A presença de hipóxia em tecido dérmico lesado estaria assim relacionada à indução de citocinas angiogênicas, síntese de proteínas de matriz extracelular e regeneração tissular (HAROON et al., 2000).

Estes efeitos da hipóxia sobre o fenótipo celular são atribuídos às mudanças adaptativas sofridas pelas células em ambiente hipóxico. Os mecanismos moleculares envolvidos nesta adaptação estão relacionados com a expressão de um fator de transcrição, o HIF-1 (hypoxic inducible transcription factor-1), reconhecido como o regulador global da resposta celular a alterações na homeostase do oxigênio, ativando a expressão de mais de 100 genes cruciais a adaptação a hipóxia (KOH; SPIVAK-KROIZMAN; POWIS, 2008). Esta proteína heterodimérica é composta de uma das três subunidades alfa (HIF-1α, HIF-2α ou HIF-3α) e uma subunidade beta. Enquanto a subunidade beta se encontra constitutivamente expressa nas células, a subunidade alfa é regulada pelos níveis de oxigênio celular e, sob condições normóxicas, é rapidamente degradada. A estabilidade de HIF-1α é inibida pela hidroxilação pós-transcricional realizada pelas enzimas dependentes de oxigênio, as prolil-hidroxilases, que levam HIF-α a uma rápida degradação proteossomal (SEMENZA, 2009). Assim, a estabilidade e a atividade de

indução da transcrição do HIF-α são negativamente reguladas pela hidroxilação dependente de oxigênio. Em hipóxia, há inibição destes mecanismos reguladores da atividade de HIF-1, permitindo estabilização e acúmulo da subunidade alfa. O acúmulo nuclear e citoplasmático de HIF-1α e sua complexação com HIF-1β desencadeia ativação e expressão de diversos genes envolvidos principalmente nos processos de angiogênese, metabolismo da glicose, proliferação celular e apoptose (CROWTHER et al., 2001; LU et al., 2005; SEMENZA, 2001).

As características dos dois modelos experimentais de hipóxia, tumores e úlceras induzidas, tais como predominância de compartimentos hipóxicos, acidose, neovascularização e presença de macrófagos, permitem generalizações quanto ao importante papel regulador da hipóxia no fenótipo celular e nos processos fisiopatológicos (FELDMEIER et al., 2003). Os focos teciduais de infecção também são caracterizados pelos baixos níveis de oxigênio e glicose (DEHNE; BRÜNE, 2009; ZINKERNAGEL; JOHNSON; NIZET, 2007). Recentes trabalhos avaliaram o efeito da hipóxia ou o papel do principal fator transcricional da resposta celular a hipóxia, HIF-1α, em processos patológicos resultantes da presença de microrganismos (CRAMER et al., 2003; KEMPF et al., 2005; PEYSSONNAUX et al., 2005; RIESS et al., 2004; RUPP et al., 2007; SPEAR et al., 2006; WAKISAKA et al., 2004). Assim, um modelo experimental que apresenta a variável "infecção" acrescenta importantes informações sobre as modificações fenotípicas de células, principalmente macrófagos ativados com antígenos microbianos ou infectados com patógenos, em ambiente hipóxico. Os modelos *in vivo* e *in vitro* da leishmaniose têm sido utilizados em nosso laboratório para estes estudos.

1.2. Os macrófagos e a hipóxia

Os macrófagos são fagócitos profissionais multifuncionais e, dependendo do estado de ativação e da sua localização, apresentam antígenos, secretam uma variedade de fatores

regulatórios, enzimas e citocinas e têm atividades anti-tumoral e microbicida (ADEREM; UNDERHILL, 1999; FUJIWARA; KOBAYASHI, 2005; GLAROS; LARSEN; LI, 2009; GORDON, 2007; NAITO, 2008). Estas células acumulam-se no centro ou nas adjacências de áreas hipóxicas e pouco vascularizadas de tecidos lesados (revisto por LEWIS et al., 1999; SIVEEN; KUTTAN, 2009).

A maioria dos trabalhos de análise das proteínas expressas em hipóxia com diferentes tipos celulares tais como células tumorais, citotrofoblastos e cardiomiócitos (revisto por KUMAR; KLEIN, 2004), têm demonstrado alterações em três grupos principais de proteínas: as proteínas do metabolismo energético (enzimas glicolíticas), fatores transcricionais (HIF-1α) e proteínas relacionadas ao estresse (HSP70 e cicloxigenases). Nos trabalhos realizados com macrófagos submetidos a hipóxia demonstrou-se modulação positiva da expressão de iNOS, apesar da baixa concentração de NO e aumento da produção de VEGF (ALBINA et al., 1995). O fator de transcrição HIF-1α (hypoxic inducible factor-1) é expresso em macrófagos murinos cultivados em hipóxia e em macrófagos associados a áreas avasculares de vários tipos de carcinomas (KAUFMAN et al., 2004; SEMENZA, 2001). Em trabalho recente do nosso grupo de pesquisa, demonstramos que macrófagos infectados com o parasita *Leishmania amazonensis* e cultivados em hipóxia apresentam alterações na expressão de proteínas do choque térmico, principalmente HSP70 (DEGROSSOLI et al., 2004). Apesar de ocorrer correlação positiva entre a expressão de HSP70 em astrócitos e cardiomiócitos e dano celular em hipóxia (IWAKI et al., 1993; UEHARA et al., 1999), nos macrófagos a relação é inversa. Estas células são resistentes a hipóxia e reduzem significativamente a expressão de HSP70, sugerindo adaptação dos fagócitos mononucleares a este microambiente (DEGROSSOLI et al., 2004). Posteriormente demonstramos que macrófagos J774 expostos a períodos prolongados de hipóxia severa resulta em seleção de células com fenótipo associado a modulação da expressão de HSP70, produção de

TNF-α e óxido nítrico (NO) e reduzida susceptibilidade a infecção por *L. amazonensis* (DEGROSSOLI et al., 2007).

Há vários efeitos da hipóxia nas funções dos macrófagos. Estudos realizados *in vitro* demonstraram que os macrófagos podem se adaptar a hipóxia, resistindo a apoptose, alterando o metabolismo para uma via glicolítica anaeróbica e os processos de endocitose/fagocitose, produzindo moléculas de adesão, expressando a enzima iNOS e modificando o metabolismo colesterol (LEEPER-WOODFORD; DETMER, 1999; LEWIS et al., 1999; MELILLO et al., 1996; MATSUMOTO et al., 2000). Períodos prolongados de hipóxia induzem secreção de TNF, sugerindo que funções inflamatórias e citotóxicas dos macrófagos estão aumentadas após a isquemia (LAHAT et al., 2003; LEEPER-WOODFORD; DETMER, 1999).

Em relação ao potencial fagocítico, macrófagos alveolares e células de Kupffer expostos a hipóxia *in vitro* e *in situ* mostraram capacidade reduzida para ingerir partículas de poliestireno e carbono coloidal (REICHNER et al., 2001; SIMON; AXLINE; PESANTI, 1981; TE KOPPELE et al., 1991). Leeper-Woodford et al. (1993) avaliaram alguns parâmetros como aderência, concentração de ATP e viabilidade. Nestes experimentos, apesar das células de Kupffer em hipóxia apresentarem índices fagocíticos reduzidos para hemácias fixadas, elas eram viáveis, aderentes, tinham a capacidade de recuperar sua função fagocítica e de aumentar a produção de ATP aos níveis normais quando cultivadas novamente em ambiente normóxico. Em contraste, Anand et al. (2007) e Acosta-Iborra (2009) observaram que macrófagos peritoneais cultivados em hipóxia mostraram aumento na fagocitose de hemácias e beads de latex comparado a normóxia. Um efeito interessante da hipóxia na retenção de partículas é a descrição de que macrófagos que fagocitaram hemácias em ambiente normóxico e depois foram cultivados em pO_2 reduzida apresentavam as hemácias em sua superfície, sugerindo um processo de exocitose (LEEPER-WOODFORD et al., 1993).

Em relação a funcionalidade imune de macrófagos em hipóxia, há trabalhos demonstrando que estas células cultivadas em pO_2 reduzida produzem quantidades altas de linfocinas pró-inflamatórias e de fatores quimiotáticos, tais como IL-1, TNF, IL-6, IL-8 e MIP-1α (macrophage inflammatory protein-1 alpha). Albina et al. (1995) mostraram que, apesar da supressão da síntese de NO (óxido nítrico) devido à falta de um de seus substratos, o oxigênio, macrófagos em hipóxia têm aumentado o metabolismo da arginina, a atividade arginase e a expressão da enzima iNOS. Os autores sugerem que a hipóxia, se transitória, deve "primar" macrófagos e que, durante a reoxigenação, estas células responderiam mais eficientemente a estímulos externos.

Com relação a função "apresentadora de antígeno" (APC) de macrófagos, trabalhos prévios sugeriram que esta função estaria alterada durante processos hipóxicos (LAHAT et al., 2003; MURATA et al., 2002). Estes autores demonstraram que macrófagos e células dendríticas murinas produzem grandes quantidades de interferon gama sob condições hipóxicas (MURATA et al., 2002) e que a expressão de CD80 (molécula co-estimulatória de superfície, envolvida na apresentação de antígenos) está reduzida em monócitos humanos cultivados em hipóxia (LAHAT et al., 2003). Recentemente foi relatado que macrófagos em hipóxia aumentam a expressão de moléculas co-estimulatórias e moléculas envolvidas na apresentação de antígeno, como CD40, CD86 e MHC de classe I, ativando mais eficientemente células T na presença de um antígeno específico (ACOSTA-IBORRA et al., 2009).

O nosso grupo de pesquisa avaliou o efeito da baixa tensão de oxigênio em macrófagos durante um processo infeccioso. Os nossos trabalhos indicaram que a hipóxia altera o fenótipo de macrófagos em relação à infecção com *L. amazonensis*, tornando-os mais resistentes à proliferação intracelular deste parasita (COLHONE et al., 2004; DEGROSSOLI et al., 2004). Os

mecanismos envolvidos na resistência dos macrófagos em ambiente hipóxico foram objetos de estudo deste trabalho.

1.3. A leishmaniose

A leishmaniose é um grupo de doenças causado por um protozoário do gênero *Leishmania*, parasita de macrófagos. As formas da doença variam desde lesões cutâneas simples, que se curam espontaneamente, a leishmaniose visceral que, quando não tratada, é fatal. Estas variações dependem da espécie de *Leishmania*, do status imune do hospedeiro e de outros fatores não conhecidos (DAVISON, 2005; HERWALDT, 1999; MURRAY et al., 2005). As formas amastigotas localizam-se no fagolisossomo de macrófagos, onde se multiplicam, rompem a célula hospedeira e infectam outros macrófagos. Utilizando-se camundongos infectados com *L. major* e *L. amazonensis*, diversos mecanismos de susceptibilidade e resistência foram descritos, como a estimulação de diferentes tipos de linfócitos T CD4 (Th1/Th2), as linfocinas ativadoras de macrófagos (IFN-γ e IL-12) e as espécies reativas de oxigênio e nitrogênio produzidas por macrófagos, tóxicas ao parasita (AWASTHI; MATHUR; SAHA, 2004; GIORGIO et al., 1998; LINARES et al., 2001; SOLBACH; LASKAY, 2000). Devido à facilidade da utilização dos modelos *in vitro* e *in vivo* da infecção com *Leishmania*, a eficiência de vários compostos e de protocolos de vacinação tem sido testada, mas ainda não há drogas mais eficientes ou vacinas disponíveis (revisto por HANDMAN, 2001; MURRAY et al., 2005). Esta parasitose continua a figurar como um importante problema de saúde pública, com uma incidência estimada em 1,5 a 2 milhões de novos casos por ano (SHAW, 2007; WORLD HEALTH ORGANIZATION). No Brasil, a progressão de casos tem sido observada e, no Estado de São Paulo, nos últimos anos, um número significativo de infecções tem ocorrido (CAMARGO-NEVES; GOMES ADE; ANTUNES, 2002; GIORGIO et al., 1996;). A resistência às drogas utilizadas tradicionalmente na

terapia, aliada aos efeitos colaterais destes compostos, fazem com que alternativas ao tratamento sejam estudadas (HERWALDT, 1999; MURRAY et al., 2005).

A *L. amazonensis*, espécie que utilizamos em nossos estudos, provoca lesões cutâneas em humanos que respondem ao tratamento ou podem curar-se espontaneamente (GRIMALDI; TESH, 1993). Em linhagens murinas susceptíveis, as lesões são caracterizadas por um grande número de amastigotas em divisão, migração de células inflamatórias (MCELRATH et al., 1987), infecções secundárias com bactérias anaeróbicas e aeróbicas e mudanças no fluxo sanguíneo tecidual (EL-ON; SNEIER; ELIAS, 1992; GIORGIO et al., 1998), que resultam em isquemia local e hipóxia (ARRAIS-SILVA et al., 2005).

Estudos realizados por vários autores nos modelos *in vitro* (em normóxia) têm mostrado que macrófagos sofrem mudanças após a infecção com *Leishmania*, como por exemplo, alterações na expressão de proteínas e síntese de citocinas, inibição da produção de metabólitos tóxicos do oxigênio e nitrogênio e modulação da apoptose (ALEXANDER; SATOSKAR; RUSSELL, 1999; BOGDAN; RÖLLINGHOFF, 1999; LINARES et al., 2001; OSORIO Y FORTÉA et al., 2007; OVERATH; AEBISCHER, 1999; STAFFORD; NEUMANN; BELOSEVIC, 2002). As avaliações destes parâmetros em células infectadas cultivadas em microambiente hipóxico, situação que ocorre nas lesões (ARRAIS-SILVA et al., 2005), é um dos focos da nossa pesquisa.

1.4. O modelo *in vitro* da leishmaniose e a hipóxia

Uma das principais premissas que nos levaram a avaliar a influência da hipóxia durante a infecção com *Leishmania* foram as observações de que lesões cutâneas em modelo murino susceptível têm características de tecido hipóxico, tais como grande número de amastigotas em divisão, migração de células inflamatórias, infecções secundárias com bactérias aeróbicas e

anaeróbicas e mudanças no fluxo sangüíneo tecidual (EL-ON; SNEIER; ELIAS, 1992; GIORGIO et al., 1998). De fato, recentemente, o grupo de pesquisa demonstrou a expressão do marcador de hipóxia e fator de transcrição HIF-1α em lesões de camundongos BALB/c infectados com o parasita (ARRAIS-SILVA et al., 2005).

Os efeitos da hipóxia durante a infecção *in vitro* também foram avaliados. O nosso trabalho demonstrou que a hipóxia altera o fenótipo de macrófagos em relação à infecção com *L. amazonensis*. Macrófagos de linhagens e culturas primárias humanas e murinas mantidas em microambiente hipóxico (6% O_2, 5% CO_2, equilibrado com N_2), quando comparados às culturas normóxicas, reduzem a carga parasitária em aproximadamente 50% (COLHONE et al., 2004). A cinética da infecção indicou que este fator microambiental não deprime a fagocitose de amastigotas, mas altera o fenótipo de macrófagos infectados para uma condição "ativada/estimulada". Macrófagos pré-expostos a esta condição microambiental não são metabolicamente comprometidos e recuperam a capacidade de célula hospedeira do parasita (DEGROSSOLI et al., 2004). Nossos dados sugerem que o fenótipo de resistência à infecção observado em condições de hipóxia (COLHONE et al., 2004) é semelhante àquele encontrado em culturas tratadas com ativadores clássicos (interferon gama e LPS, em normóxia) (GREEN et al., 1990; LINARES et al., 2000). As culturas celulares em hipóxia também mostraram baixa produção de nitrito em seus sobrenadantes, sugerindo um mecanismo "leishmanicida" ou modulador da infecção, independente da produção de intermediários do nitrogênio (COLHONE et al., 2004). No presente trabalho de tese avaliamos as modificações dos macrófagos submetidos a hipóxia com a finalidade de explicar os efeitos leishmanicidas da hipóxia.

2. OBJETIVOS

O objetivo geral deste projeto foi contribuir para a compreensão do papel terapêutico e/ou deletério da hipóxia em infecções intracelulares como a leishmaniose.

Os objetivos específicos são:

1) Avaliar os possíveis mecanismos envolvidos na redução da infecção em macrófagos infectados com *L. amazonensis* e cultivados em hipóxia (COLHONE et al., 2004; DEGROSSOLI et al., 2004):

a) testando a hipótese de que o NO não está envolvido nos efeitos leishmanicidas de macrófagos em hipóxia, após a avaliação da resistência de macrófagos originados de camundongos knockout para a enzima iNOS (não produtores de NO) ao parasita em hipóxia;

b) analisando a produção de intermediários reativos do oxigênio;

c) analisando a produção das citocinas TNF, IL-6, IL-10, IL-12;

d) testando a possibilidade de estes macrófagos alterarem o processo fagocítico ou realizarem exocitose em hipóxia;

e) analisando a produção de ATP pelos macrófagos em hipóxia;

f) avaliando se há apoptose de amastigotas intracelulares em macrófagos submetidos a hipóxia

2) Avaliar se os macrófagos cultivados em hipóxia e infectados com *L. amazonensis* sofrem alteração na expressão do fator de transcrição HIF-α (hypoxia inducible factor-α).

3) Avaliar a capacidade de apresentação de antígeno de macrófagos pulsados com antígeno (proteínas de *L. amazonensis)* em ambiente hipóxico.

3. APRESENTAÇÃO DO ARTIGO CIENTÍFICO

No decorrer do trabalho de tese parte dos resultados foi publicado na forma de artigo científico, em anexo. A seguir fazemos uma breve descrição do trabalho.

1. Degrossoli A, Bosetto MC, Lima CBC, Giorgio S. 2007. *Expression of hypoxia-inducible factor 1α in mononuclear phagocytes infected with* Leishmania amazonensis*.* Immunol Letters, 114: 119-25. (Anexo).

O HIF-1α pode ser induzido em diferentes tipos celulares por estímulos não hipóxicos como fatores de crescimento, citocinas, óxido nítrico, lipopolissacarídeos e uma variedade de microrganismos infecciosos. Neste artigo, através da técnica de imunofluorescência, relatamos a habilidade de fagócitos mononucleares (macrófagos murinos e humanos e células dendríticas humanas) em expressar HIF-1α quando parasitados com *Leishmania amazonensis*. A expressão de HIF-1α nestes fagócitos não se deve ao fato destas células se tornarem hipóxicas após a infecção com *L. amazonensis*, pois não foi observada reação positiva para o marcador de hipóxia pimonidazol. Macrófagos infectados com *L. amazonensis* e tratados com cloreto de cádmio, um inibidor de HIF-1α, mostraram uma redução na sobrevivência do parasita. Estes resultados indicam que HIF-1α é importante na respostas adaptativas dos fagócitos mononucleares infectados com o parasita *L. amazonensis*.

4. RESULTADOS NÃO PUBLICADOS

4.1. MANUSCRITO

Title: Analyses of functional alterations of macrophages cultured in hypoxia and infected with *Leishmania amazonensis*

Authors: Adriana Degrossoli[1], Wagner Welber Arrais-Silva[1], Marcelle Carolina Colhone[1], Fernanda Ramos Gadelha[2] and Selma Giorgio[1]

1. Departamento de Biologia Animal, Instituto de Biologia, Universidade Estadual de Campinas, Caixa Postal 6109, 13083-970, Campinas, São Paulo, Brazil

2. Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas, Caixa Postal 6109, 13083-970, Campinas, São Paulo, Brazil

Corresponding Author: Selma Giorgio

Departamento de Biologia Animal, Instituto de Biologia, Universidade Estadual de Campinas, Caixa Postal 6109, 13083-970, Campinas, São Paulo, Brazil

Fax number: 55 19 35216282

Phone number: 55 19 35216287

e-mail: sgiorgio@unicamp.br

ABSTRACT

Regions of low oxygen tension (hypoxia) are common features of inflamed/infected tissues. Macrophages, cells involved in the clearance of microorganisms from infected tissues, are influenced by oxygen tension. Recently we have shown that macrophages exposed to hypoxia and infected with *Leishmania amazonensis* amastigotes are able to reduce intracellular parasitism. However, the mechanisms contribute to the resistance of macrophages to *L. amazonensis* infection under hypoxic microenvironmental are not known. In this study we investigated modifications of infected macrophages in hypoxia, such as NO and ROS synthesis, cytokines production, exo/phagocytosis, ATP release, HIF-2α expression, and whether apoptosis occurred in intracellular amastigotes. Our results indicate that hypoxia does not induce the synthesis of NO in macrophages infected with *L. amazonensis* as well as iNOS expression, and iNOS knockout macrophages lacking NO synthesis are still able to reduce infection when cultured in a hypoxia. Although noninfected macrophages produce more ROS in hypoxia than in normoxia, *L. amazonesis*-infected macrophages show similar levels of ROS in normoxic and hypoxic conditions. Antioxidants NAC (ROS scavenger) and Ebselen (glutathione peroxidase mimic) inhibit the leishmanicidal effect of hypoxia, indicating ROS may be important to the effect of hypoxia on leishmanial infection. The cytokines TNF-α, IL-12 and IL-10 releases was similar in normoxia and hypoxia by infected macrophages. Although hypoxia inhibits the phagocytosis of inert particles or fixed parasite, it does not affect *L. amazonensis* entry into macrophages. Hypoxic treatment does not also induce the exocytosis of internalized particles by macrophages. Infected macrophages show similar levels of ATP in normoxia and hypoxia and apoptosis-like death in intracellular amastigotes did not occur in hypoxic conditions. HIF-2α immunoreactivity is elevated in nuclei of macrophage infected with *L. amazonensis.* Thus, with exception of ROS, NO, cytokines, phago/exocytosis and energetic metabolism of macrophages

are not related to the anti-*Leishmania* activity of hypoxia. Furthermore, we can speculate HIF-2α is involved in the phenotype changes of infected macrophages in hypoxia.

INTRODUCTION

Regions of low oxygen tension (hypoxia) are common features of tumors, wounds, atherosclerotic lesions, and inflamed/infected tissues (1-4). Macrophages, cells involved in the clearance of microorganisms from infected tissues, antigen processing/presentation and, angiogenesis, are influenced by oxygen tension (5). Experimental hypoxia has been reported to influence macrophage adaptations, including cytokine secretion, expression of cell surface markers, migration, pinocytosis, and phagocytosis of inert particles (6-12). We have studied the influence of low oxygen tension on macrophage response to parasite stimulus (13-17). Macrophages exposed to hypoxia and infected with *Leishmania amazonensis* were able to reduce intracellular parasitism (13,14). Moreover, macrophage exposure to hypoxia reduce expression of the 70-kD heat shock protein (HSP70) (14,16) and induce TNF-α release (16). The macrophage alterations induced by hypoxia is related to the hypoxia-inducible factor 1α (HIF-1α), since we have reported mononuclear phagocytes (macrophages and dendritic cells) expressed this transcription factor when cultured in hypoxia (17). The *Leishmania* protozoan is an intramacrophage parasite that causes chronic human diseases ranging from localized to diffuse cutaneous infections (18,19). 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 (18,20). These lesions regenerate over months or years (18). Diffuse cutaneous leishmaniasis is associated with cutaneous metastases from the initial skin lesion (18,19). Several characteristics of leishmanial lesions in humans and in animal models, such as microcirculatory impairment,

metabolic demand for leukocyte infiltration into infected tissues, parasite proliferation, secondary bacterial infection and HIF-1α expression (15,18,21-24) are strong indications of a hypoxic microenvironment in lesions that may play a role in the outcome of infection. Although we have demonstrated that macrophages exposed to hypoxia and infected with *L. amazonensis* reduce intracellular parasitism (13,14,16,17), the mechanisms that contribute to the resistance of macrophages to *L. amazonensis* infection under hypoxic microenvironmental are poorly understood. In this study we investigated modifications in infected macrophages in hypoxia, such as ROS synthesis, cytokines production, exo/phagocytosis, ATP release, HIF-2α expression, and whether apoptosis occurred in intracellular amastigotes, in order to elucidate mechanisms by which macrophages are able to control *L. amazonensis* infection in hypoxia.

MATERIALS AND METHODS

Reagents

Cell Culture and Parasites

The murine macrophage cell line, J774, obtained from the American Type Culture Collection (Rockville, Md., USA) 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% fetal calf serum (Nutricel, Campinas, Brazil) at 37 °C in 5% CO_2, 5% O_2 and balanced N_2 (16). Primary mouse macrophages were obtained from normal BALB/c mice by peritoneal lavage (24,25). *Leishmania amazonensis* (MHOM/BR/73/M2269) amastigote forms were isolated from active skin lesions of BALB/c mice as described previously (25). The parasites were suspended in RPMI 1640 medium and used immediately after isolation.

Norrnoxic and Hypoxic Conditions

Hypoxic cell culture conditions were established as described previously (14). The cell cultures were placed in a gas-tight modular chamber (Billups-Rothenberg, Del Mar, CA); the chamber was gassed for at least 15 min at a flow rate of 2 L/min using certified gases containing O_2, CO_2 and N_2 (White-Martins Gases, Rio de Janeiro, Brazil) and placed in a 37°C temperature-controlled incubator. The percentage of O_2 was verified by measuring the outflow of gas at the end of the initial flushing period and then at 24 h intervals using a Fyrite apparatus (Bacharach, Inc., Pittsburgh, PA). The oxygen tension in the culture medium under hypoxic conditions was 7 or 37 mmHg and it was 150 mmHg under normoxic conditions (O_2 Analyzer YSI/53, Yellow Springs Instruments Inc., Yellow Springs, OH). In all experiments, cell exposure to <1% 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 medium pH was 7.4 and did not change significantly during the course of the experiments.

Macrophage Infection, and Activation and Assessment of Intracellular Parasites

Peritoneal macrophages were infected with *L. amazonensis* amastigotes (3:1 parasites/host cell) for 1 h, as previously described (13). After the interaction period, the cultures were washed to remove extracellular parasites and fresh medium was added to the cell culture. J774 macrophages were infected with *L. amazonensis* amastigotes (10:1 parasites/host cell) for 24 h, as previously described (16). Intracellular parasite destruction was assessed by morphological examination. Briefly, for evaluating the percentage of infected macrophages and the number of amastigotes per macrophage, cells on coverslips were stained with Giemsa (24). Intracellular amastigotes, which are exclusively localized in parasitophorous vacuoles (26), were examined microscopically at a magnification of 1000 ×. About 600 cells were counted per triplicate coverslip. For the experiments of macrophage activation, cells were treated with 200 ng/mL

recombinant mouse gamma interferon (IFN-γ) and 100 ng/mL *Escherichia coli* lipopolysaccharides (LPS; Sigma), 8 h before the *Leishmania* infection (27).

Immunoblotting Analyses

After different treatments, cells were scraped from culture flasks, checked for viability, and then rinsed twice with PBS. Lysis buffer (62.5 mM Tris-HC1, pH 6.8, 69 mM SDS, 10% glycerol, 2% 2-mercaptoethanol, 34 mM ethylenediaminetetraacetic acid, 2 µg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride; Amersham Pharmacia Biotech, Piscataway, N.J., USA) was added to the cell pellets. Proteins were denatured at 95°C for 3 min, eletrophoresed on a 10% SDS-PAGE (polyacrylamide) gel system (Thermo EC, Holbrook, N.Y., USA) and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech). After blotting, membranes were incubated with mouse monoclonal anti-inducible nitric oxide synthase (anti-iNOS) antibody (Sigma) diluted 1:1000. The secondary antibody consisted of peroxidase-conjugated rabbit anti-mouse IgG (Sigma), and it was detected using the enhanced chemiluminescence (ECL) Western Blotting Analysis system (Amersham Pharmacia Biotech).

Nitrite Assay

Nitrite content in the supernatants of macrophage cultures was measured by the Griess method (28) using a colorimetric assay kit (Sigma), as previously described (16).

DCF Assay for ROS

Formation of ROS was determined by the use of the fluorescent probe 2,7-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Invitrogen, USA), as previously described (29). Peritoneal macrophages were plated in microplate (2×10^5 cells/well). After 24 h the macrophages were infected with *L. amazonensis* for 1 h, 37ºC. Then, the complete medium was removed and macrophages were incubated with 10 µM DCFH-DA in buffered solution with 10 mM phosphate, 5 mM glucose and 140 mM NaCl (ph 7,0) for 15 min at 37ºC. Thereafter, 10

µg/mL phorbol 12-myristate 13-acetate (PMA; Sigma) were added to the cells and plates were maintained in normoxia or hypoxia for 1.5 h or 3 h (modified protocol from 30 and 31). The fluorescence was measured by a multiwall fluorescence plate reader (Synergy HT, Biotek, USA), at an excitation wavelength of 480 ± 10 nm and an emission wavelength of 520 ± 10 nm. The relative fluorescence was calculating using the following formula: $Ft/Fc \times 100$, where *Ft* is the fluorescence of sample test and *Fc* is the fluorescence of macrophages in normoxia (control).

Antioxidants

The following antioxidants were used to inhibit ROS formation by macrophages: *N*-acetylcysteine (NAC; Sigma) or 2-Phenyl-1,2-benzisoselenazol-3(2H)-one (Ebselen; Calbiochem, USA). NAC was diluted in deionized water sterile and added to the cultures at final concentration of 5 mM for 2 h, at 37ºC, before the treatments of hypoxia or infection (32). The compound Ebselen was diluted in DMSO and 40 µM Ebselen (0,02% DMSO) were added to the cultures in the beginning of the experiments (33).

Cytokines assay

The IL-6, IL-10 and IL-12 concentration in the macrophage cultures supernatants was determined using an enzyme-linked immunosorbent assay (ELISA) kit (Biosource, USA), according to the manufacturer's instructions. 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 (9,16,34). The sensitivity of the L929 cell line was determined by using recombinant murine TNF-α. After 24 h 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 (Synergy HT, Biotek, USA). The cytotoxicity effects caused by supernatants containing TNF-α activity were calculated using the following formula: Cytoxicity (%) = $[OD_{540}control - OD_{540}test]/OD_{540}control \times 100$, where *control* and *test* represent absorption of L929 plus medium

and L929 plus supernatants, respectively (34). One unit of TNF activity equals 50% L929 citotoxicity (9).

Phagocytosis and Exocytosis Assays and Preparation of IgG-Opsonized Microspheres

Phagocytosis assays were performed using non-opsonized or opsonized particles. When opsonized particles were used, FluoSpheres® carboxylate-modified microspheres, yellow-green fluorescent (505/515 FluoSpheres®, 2 µm in diameter) were obtained from Molecular Probes, Invitrogen and were coated with IgG using the method of Cannon and Swanson (35). Briefly, microspheres were incubated with 10 mg/ml bovine serum albumin (BSA) (Sigma) in phosphate-buffered saline (PBS) for 1 h at 37ºC. The BSA-coated microspheres were then washed three times with PBS and incubated with the mouse anti-BSA serum (1:250 dilution) (Sigma) for 30 min at 37ºC. The IgG-coated microsphere suspension was then cooled to 4ºC for 10 min, washed three times with PBS, and resuspended in complete medium. Microspheres were enumerated using a hemocytometer and diluted to a final concentration of 2×10^7 microspheres/ml. In the phagocytosis assays, peritoneal macrophages were plated in microplates at a density of 10^5 cells/well and incubated overnight at 37 °C. The macrophages were exposed to 100 µL non-opsonized FITC-labeled *Escherichia coli* K-12 bioparticles (1mg/mL) or opsonized microspheres for 1 h in normoxia or hypoxia conditions. Phagocytosis was assayed by measuring the fluorescence intensity of the cells at an excitation wavelength of 480 ± 10 nm and an emission wavelength of 520 ± 10 nm using a fluorescence spectrometer microplate reader (Synergy HT, Biotek, USA. The fluorescence of particles bound to the surface of the cells was quenched by adding trypan blue to cell cultures at 10 mM final concentration for 1 min. The relative phagocytosis was calculated using the following formula: $Ft/Fc\times100$, where *Ft* is the fluorescence of sample test and *Fc* is the fluorescence of macrophages in normoxia (control). In the exocytosis assay, macrophages were exposed to 100 µL FITC-labeled *Escherichia coli* K-12

bioparticles (1mg/mL) for 1 h in normoxia conditions. Thereafter, extracellular bioparticles were removed and fluorescence was measured. Macrophages were further incubated in normoxia or hypoxia for 1 and 24 h and the fluorescence was measured after each time of incubation. The relative fluorescence was calculated using the following formula: $Ft/Fc \times 100$, where *Ft* is the fluorescence of sample test and *Fc* is the fluorescence of macrophages in normoxia (control) in the initial time.

L. amazonensis phagocytosis were performed with freshly or fixed amastigotes. Parasites from footpad lesions of BALB/c mice were fixed for 60 min at 4ºC with 0.5% glutaraldehyde (Merck, USA) in PBS solution at 10^8 parasites/mL (36). The fixed parasites were washed three times with PBS, resuspended in PBS at 10^9 parasites/mL and maintained at 4ºC until use. Peritoneal macrophages were plated in 13-mm glass coverslips at a density of 5×10^5 cells/well and incubated overnight at 37 °C. Macrophages were incubated with living amastigotes (3:1 parasites/host cell) or fixed amastigotes (10:1 parasites/host cell) for 1 h in normoxia or hypoxia. After the interaction period, the cultures were washed to remove extracellular parasites and cells on coverslips were stained with Giemsa (24). The phagocytosis was quantified by morphological examination, evaluating the percentage of infected macrophages and the number of amastigotes per macrophage microscopically at a magnification of 1000×. About 600 cells were counted per triplicate coverslips.

TUNEL Assay

In situ detection of DNA fragments by terminal deoxyribonucleotidyltransferase (TdT)-mediated dUTP nick ending labeling (TUNEL) was performed using Fluorescein FragEL™ DNA Fragmentation Detection Kit (Calbiochem) following the manufacturer's instructions. Infected macrophages on copverslips were fixed for 10 min with 4% paraformaldehyde and washed 3× 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). All images were captured and analyzed with a digital camera (Nikon DXM1200-F) and imaging software (ACT-1, Nikon).

ATP Assay

Cellular ATP content was determined using a luciferase-based ATP assay kit ATPlite (Perkin-Elmer, Life and Analytical Sciences, Boston, MA, USA), according to 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, USA) after mixing and 10 minutes dark adaptation of the plate. ATP standards and blanks were incorporated in each reading.

HIF-2α Immunofluorescence

Cells attached to the slide-chambers were fixed for 10 min with 4% paraformaldehyde and washed 3× in PBS. The cells were permeabilized with 1% Tween 20 and then washed 2 × in PBS. Nonspecific binding sites were blocked with 3% BSA (Amresco, Solon, OH) for 30 min. The cells were then incubated with rabbit anti-HIF-2α antibody diluted 1:400 (a gift from Dr. Wagner W. Arrais-Silva) overnight at 4ºC in a wet room. The cells were washed 3 × 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. The cells were washed 3 × in PBS + 0.1% Tween 20 and mounted with DAPI-containing DABCO mounting media (Sigma). The cells were visualized under a Nikon Eclipse 50*i* fluorescence microscope (Nikon Inc., Melville, NY). All images were captured and analyzed with a digital camera (Nikon DXM1200-F) and imaging software (ACT-1, Nikon).

Statistical Evaluation

All experiments, except for immunofluorescence analyses, were repeated at least 3 times, and the results are expressed as the mean ± SD. The immunofluorescence experiments were repeated at least 10 times. Data obtained under different conditions (hypoxia and normoxia; noninfection and infection) were analyzed statistically by Student's *t*-test, with levels of significance set at $p \leq 0.05$ for in vitro assays.

RESULTS

NO production and iNOS expression

Macrophages from two different sources, J774 cell line and primary macrophages obtained from the peritoneum of mice, are efficiently infected with *Leishmania* in normoxia for 24 h, showing a significantly high number of infected macrophages (47% for J774 cultures and 72% for peritoneal macrophage cultures) as well as intracellular parasites (5.7 for J774 cells and 7.8 for peritoneal macrophages) (14). However, in cultures subjected to hypoxia for 24 h, there was a significant reduction in the percentage of infected macrophages (21% for J774 cultures and 32 % for peritoneal macrophages) and also in the number of parasites inside the cells (2.8 for J774 cells and 2.3 for peritoneal macrophages) when compared with the normoxic condition (14). Interestingly (in an interesting manner) the rate of reduction of infection observed in cells under hypoxia was comparable to that obtained for macrophages stimulated with IFN-γ and LPS, a well-known system which activates killing of *Leishmania* in macrophages (27,37,38).

To test whether macrophage resistance to *L. amazonensis* infection is due the nitric oxide, one of the major effector molecules for lymphokine-macrophage killing of *Leishmania* spp (38-40), we examined the nitric oxide production, as well as iNOS expression. High levels of nitrite were induced in J774 macrophages cultures stimulated with IFN-γ+LPS in normoxic conditions

(Table 1), as previously reported (27). Nonstimulated macrophages did not produce nitrite in normoxia as well in hypoxia (Table 1). It should be noted that there is no nitrite production in J774 macrophages infected with *L. amazonensis* in normoxia and hypoxia (Table 1). The question of whether the lack of nitrite production in hypoxic cultures is related to the lack of iNOS expression in macrophages was addressed by measuring the levels of iNOS protein in the cell lysates. As shown in the Figure 1, J774 cells treated with IFN-γ+LPS showed iNOS expression (Fig. 1, lane 1). There was no detectable iNOS protein in nonstimulated macrophage cultured under normoxic or hypoxic conditions (Fig 1, lanes 2 and 4) as well in macrophage infected under normoxic or hypoxic conditions (Fig. 1, lanes 3 e 5). Thus, these data indicate no correlation between nitrite production and the reduction of infection in macrophages under hypoxia. To confirm the hypothesis that nitrite is not related to the anti-leishmanial activity of hypoxia, we also evaluated the effect of hypoxia on infection of iNOS knockout macrophages, comparing to the wild type macrophages. Both cell systems were efficiently infected with *L. amazonensis* under normoxia (Fig. 2), showing a significantly high number of infected macrophages (61±5.6% for wild type culture and 48±0.6% for knockout culture) as well as intracellular parasites (4,4±.05 for wild type macrophages and 4,5±0.6 for knockout macrophages). However, in cultures subjected to hypoxia for 24 h, there was a significant reduction of the percentage of infected macrophages (35±8.2% for wild type and 28±6.7% for K.O.) and also in the number of parasites inside the cells (3±0.5 for wild type and 3.2±0.06 for K.O.). These data demonstrated that iNOS knockout macrophages also reduce the infection with *L. amazonensis* under hypoxia. Taken together, these data indicates that macrophage resistance to *L. amazonensis* infection in hypoxic conditions is not related to nitric oxide production.

ROS production and macrophage infection with antioxidants

Other molecules involved in the lymphokine-macrophage killing of *Leishmania* spp, besides reactive nitrogen intermediates (RNI), are the reactive oxygen species (ROS) produced by oxidative burst (41,42). Then, we evaluated ROS production in peritoneal macrophages infected with *L. amazonensis*. High levels of ROS were formed in peritoneal macrophages cultures stimulated with PMA during 1.5 h in normoxic conditions (Fig. 3A), as previously reported (43). In hypoxia, the production of ROS by nonstimulated macrophages (Control) or stimulated with PMA was 70% higher than macrophages in normoxia (Fig. 3A), as previously reported by Chandel et al. (30). Infected macrophages in normoxia showed increased production of ROS as compared to uninfected culture in normoxia (Fig 3A). However, ROS formation by macrophages infected with *L. amazonensis* in hypoxia was similar to uninfected macrophages in the same microenvironment (Fig. 3A). To address the question whether an increase of production of ROS in infected macrophages cultured in hypoxic microenvironment could occur later 1.5 h, we evaluated the ROS formation after 3 h of culture (Fig. 3B). The production of ROS was also similar in infected macrophages after 3 h in normoxic and hypoxic conditions (Fig. 3B). We also evaluated the effect of hypoxia on infection of macrophages cultured with antioxidants compounds, *N*-acetylcysteine (NAC), that maintain reduced glutathione levels, increasing ROS scavenging, or 2-Phenyl-1,2-benzisoselenazol-3(2H)-one (Ebselen), that acts like peroxidase gluthatione mimetic. Peritoneal macrophages infected under hypoxia showed a 30% reduction of the percentage of infected cells (Fig 4A and C) and a 28% reduction of the number of amastigotes per cell (Fig. 4B and D) compared with normoxic conditions. Infected macrophages cultured with NAC showed similar levels of infection compared with macrophages without NAC in normoxic condition (Fig. 4A and B). Interestingly, the infection was also not reduced in macrophages infected in hypoxia and cultured with NAC (Fig. 4A and B). The percentage of infection in the

culture maintained under normoxia and treated with Ebselen was similar to the culture in normoxia without Ebselen (Fig. 4C), but the number of intracellular parasites in the normoxic culture with Ebselen was lower than normoxic culture without Ebselen (Fig. 4D). However, the level of infection was similar between normoxic and hypoxic cultures treated with Ebselen (Fig. 4C and D). Besides the similar levels of ROS production by infected macrophages in normoxic and hypoxic microenvironments (Fig. 4), the antioxidants NAC and Ebselen inhibit the leishmanicidal effect of hypoxia, suggesting the ROS are important to macrophages reduce infection under hypoxia.

Cytokines production

Cytokines have long been recognized as key elements in the host response against *Leishmania* (44). Thus, we evaluated the cytokines TNF-α, IL-6, IL-10 and IL-12 production by infected macrophages cultured in hypoxia. Macrophages were infected with *L. amazonensis* amastigotes under normoxia or hypoxia conditions or were stimulated with LPS or IFN-γ+LPS, a well-defined proinflammatory activator system (45,46), and 24 h later supernatants were collected and assayed for TNF-α bioactivity or ELISA for cytokines IL-6, IL-10 and IL-12. As shown in Figure 5A, TNF-α bioactivity in supernatants cultures IFN-γ+LPS-exposed macrophages was higher than nonstimulated macrophages cultures supernatants (Fig. 5A). When macrophages were exposed to hypoxia, TNF-α bioactivity was enhanced in nonstimulated or IFN-γ+LPS-exposed macrophages supernatants (Fig. 5A). However, the TNF-α bioactivity was not altered by hypoxia in infected macrophages (Fig. 5A). High levels of IL-6 were also induced in macrophages cultures stimulated with LPS in normoxic conditions (Fig. 5B). The macrophage cultures exposed to hypoxia presented a significantly higher IL-6 release than nontreated macrophages cultures or treated with LPS, as well as infected macrophages (Fig. 5B). The highest IL-12 production was observed in supernatant cultures of IFN-γ+LPS-exposed

macrophages (Fig. 5C). Under hypoxia, the production of IL-12 upon IFN-γ+LPS stimulation was more decreased than under normoxia (Fig. 5C). Infected macrophages produced similar quantities of IL-12 in normoxic or hypoxic conditions (Fig. 5C). There was no detectable IL-10 cytokine in nonstimulated macrophages under normoxia or hypoxia (Fig. 5D). High levels of IL-10 were induced in macrophage cultures stimulated with LPS in normoxia and hypoxia (Fig. 5D). Exposure of macrophages to hypoxia did not alter IL-10 release in all conditions (Fig. 5D). Taken together, these results indicate that hypoxia induces alteration in TNF-α, IL-6 and IL-12 production. However, *L. amazonensis* infected macrophages showed alteration in the case of IL-6 release.

Effects of Hypoxia on Phagocytosis and Exocytosis

Next, the hypothesis that macrophages under hypoxia showed reduced *L. amazonensis* infection because the uptake of parasite is impaired under hypoxia was tested. First, we analyzed the role of hypoxia on macrophage phagocytosis in general, performing assays using two different particles: opsonized and nonopsonized particles, since the phagocytic capacity of the macrophage is determined by the composite of the cell surface receptors which can be classified generally as opsonic-dependent and opsonic-independent (12). Data in Fig. 6A demonstrate that the total phagocytic capacity for opsonized particles in normoxia was significantly higher than phagocytosis of nonopsonized particles. Interestingly, the internalization of nonopsonized or opsonized particles were decreased in macrophages cultured in hypoxia compared with macrophages cultured in normoxia (3,6-fold and 2,3-fold for nonopsonized and opsonized particles, respectively; Fig. 6A). These results indicate that hypoxia promotes decreased internalization of inert particles. We also evaluated the effect of hypoxia on *L. amazonensis*-phagocytosis of macrophages, using living amastigotes and comparing to glutaraldehyde-fixed amastigotes-phagocytosis as an inert parasite. Both living and glutaraldehyde-fixed amastigotes

were efficiently internalized by macrophages (Fig. 6B and C), that showed a significantly high number of infected macrophages (35±7% for living amastigotes and 30±8% for glutaraldehyde-fixed amastigotes) as well as intracellular parasites (2,5±0,3 for living amastigotes and 2,3±0,3 for glutaraldehyde-fixed amastigotes). Macrophages infected with living amastigotes subjected to hypoxia showed similar phagocytosis levels as compared with normoxia (35±7% and 37±6% of infected macrophages and 2,5±0,3 and 2,4±0,2 intracellular parasites for normoxia and hypoxia, respectively). In contrast, macrophages exposed to hypoxia had reduced phagocytosis of glutaraldehyde-fixed amastigotes, as demonstrated by a significant reduction of the percentage of infected macrophages when compared with the normoxic condition (13±3% and 37±7%, respectively). Taken together, these data indicate that, in contrast to inert particles or fixed amastigotes, the phagocytic process of living *L. amazonensis* amastigotes by macrophages is not affected by hypoxic conditions. The effect of hypoxia on macrophage exocytosis was also assessed to test the hypothesis the low infection in hypoxic condition would be the parasite exocytosis by macrophages, a process involved in the liberation of amastigotes by macrophages (47). The two macrophages cultures had the same relative fluorescence at initial time (0 h) (Fig. 7D), indicating the two cultures had the same levels of internalized particles before they were cultured in hypoxia or normoxia conditions. After 1 h and 24 h of incubation, the macrophages cultures showed similar relative fluorescence as compared to the initial time (0 h) in normoxia as well as in hypoxia. Thus, internalized particles are maintained in macrophages cultured in hypoxia for 24 h. These results indicate that exocytic capacity of macrophages is not altered by hypoxic conditions and the reduced infection by *L. amazonensis* in this microenvironment might not be related to exocytic capacity of macrophages.

ATP production

ATP was quantified to determine whether exposing peritoneal macrophages to hypoxia for 24 h affects total cellular ATP levels (Fig. 7). ATP was determined in cell lysates by bioluminescence assay, as described in *Materials and Methods*, and data normalized to the number of the cells for each sample. Treatment of macrophages with mild hypoxia (6% O_2) alone resulted in similar ATP levels compared with control culture in normoxic conditions (Fig. 7A). *L. amazonensis*-infected macrophages also showed similar levels of ATP production when cultured in normoxic or mild hypoxic microenvironment (Fig. 8A). Macrophages exposed to severe hypoxia (<1% O_2) showed a decrease in ATP levels, as compared with normoxia conditions (Fig. 7B). However, infected macrophages in severe hypoxia produced similar quantities of ATP when compared with infected macrophages in normoxia (Fig. 7B). Taken together, these results suggest the leishmanicidal effect of hypoxia is not related to the impairment of energetic metabolism of macrophages.

DNA fragmentation on intracellular amastigotes

Infected macrophages stimulated with IFN-γ+LPS reduce *L. amazonensis* infection and this antileishmanial activity is related to induction of apoptosis-like phenomena in intracellular amastigotes (48). Similarly to IFN-γ+LPS-activated macrophages, hypoxia induces macrophages to reduce *L. amazonensis* infection. However, it is not yet established whether hypoxia can induce apoptosis-like death in intracellular *L. amazonensis*. The TUNEL technique revealed that the nuclear DNA fragmentation of intracellular amastigotes did not occurred in *L. amazonensis*-infected macrophages cultured in normoxic conditions for 24 h or 48 h (Fig. 8A and B, respectively), since these amastigotes are viable and multiply inside macrophages. The nuclear fragmentation of intracellular amastigotes was also not visible inside macrophage cultured in hypoxic microenvironment for 24 h or 48 h (Fig. 8A and B). Labeled amastigote nuclei could

only be visualized inside activated macrophages, as shown in Figure 8A. Our results thus indicate that hypoxic condition does not induce apoptosis-like death process in intracellular amastigotes.

HIF-2alpha expression

Recently we have demonstrated that HIF-1α is expressed in cultured phagocytes after an exposure to the *L. amazonensis* amastigotes or promastigotes (17). Thus, this study investigated whether *L. amazonensis* interferes with HIF-2α expression, an isoform of this transcriptional factor, in macrophages by immunofluorescence studies. As shown in Figure 9, in normoxic cultures, very weak immunofluorescence was detected in the cytoplasm and nuclei of peritoneal macrophages. As might be expected, upon hypoxic exposure, macrophages responded with a strong increase in HIF-2α expression (Fig. 9). HIF-2α was expressed more intensely in the nucleus than in the cytoplasm of macrophages. Immunofluorescence analyses revealed the presence of HIF-2α in macrophages after *L. amazonensis* infection under normoxic conditions similar to the staining pattern of hypoxic noninfected cells (Figure 9). The expression of HIF-2α in infected cells cultured in hypoxia was also apparent (Fig. 9). Taken together, these experiments indicated that *L. amazonensis* amastigote infection results in HIF-2α expression in the host cell.

DISCUSSION

Previous studies provide evidence that reduced oxygen tension significantly affects the resistance of macrophages to the *L. amazonensis* infection, since macrophages exposed to hypoxia showed a reduced percentage of infected cells and number of intracellular parasites (13,14,16,17). In the present study we evaluated functional alterations of macrophages cultured under hypoxia and investigated the mechanism by which macrophages resist to *L. amazonensis* infection under hypoxia. The fact that nitric oxide (NO) is an important active component engaged in the killing of *L. amazonensis* in lymphokine activated macrophages (39-40) shows

that the measurement of nitrite production and iNOS expression is valuable to investigate the leishmanicidal mechanism of macrophages under hypoxia. Our results indicate that hypoxia does not induce the synthesis of NO in J774 cells infected with *L. amazonensis* (Table 1) as well as iNOS expression (Fig. 1). Moreover, knockout macrophages for iNOS expression lacking NO synthesis are still able to reduce infection when cultured in a hypoxic microenvironment (Fig. 2). Thus, our data indicate no correlation between NO production and the reduction of infection in macrophages under hypoxia.

Beyond NO, cytokine activation of macrophages leads to production of reactive oxygen species (ROS) through oxidative burst that are ultimately responsible for leishmanicidal activity (42;49). Then we analyzed the participation of ROS in the anti–*L. amazonensis* activity of macrophages under hypoxia (Fig. 3). Several studies have shown higher levels of ROS production by macrophages cultured in hypoxia as compared with normoxia (30,50, 51). Our data show that macrophages, elicited or not by PMA, produce more ROS in hypoxia than in normoxia (Fig. 3), as expected (30). However, *L. amazonesis*-infected macrophages show similar levels of ROS in normoxic and hypoxic conditions (Fig. 3). Interestingly, antioxidants NAC (ROS scavenger) and Ebselen (glutathione peroxidase mimic) inhibit the leishmanicidal effect of hypoxia (Fig. 4). Our interpretation of these findings is that ROS contribute to the control of *L. amazonensis* infection by macrophages in hypoxia. ROS produced by macrophages in hypoxia could be toxic for parasites, contributing to the killing of *L. amazonensis*. Previous works have demonstrated *L. donovani* amastigotes are also killed by ROS inside macrophages (52), primarily by H_2O_2 (49). During this process ROS would be consumed and no differences of ROS production in normoxia and hypoxia in *L. amazonensis*-infected macrophages could be detected. Alternatively, ROS endogenously produced by macrophages may initiate a cascade of signaling functions inducing modifications of macrophages that contribute to killing of *L. amazonensis*.

This second hypothesis is based on reports that emphasize the role of ROS as second messengers in cell signaling (53-55). ROS directly regulate the activity of transcriptions factors, such as NF-κB (56); AP-1 (57,58); and p53 (59). The ROS could induce activation of NF-κB in macrophages, for example (53,60), which is an important transcription factor involved in the development of a protective immune response against *Leishmania*, increasing transcription of a number of different genes, including those coding for chemokines and cytokines, as well as cyclooxygenase 2 (61).

Since cytokines like TNF-α, IL-6, IL-12 and IFN-γ play an important role in the leishmaniasis, and death or survival of parasite *Leishmania* depends on balance between lymphokines produced by Th1 (protection) or Th2 (susceptibility) lymphocytes (62,63), we evaluated the cytokine production by *L. amazonensis*-infected macrophages. Previous studies reported that macrophages in low oxygen tension produce high quantities of pro-inflammatory lymphokines as IL-1, TNF-α, IL-6, IL-8 and MIP-1α (64,65). Our results have shown hypoxia induces alterations in TNF-α, IL-6 and IL-12, but not in IL-10 production (Fig. 5). However, TNF-α, IL-12 and IL-10 releases was similar in normoxia and hypoxia by infected macrophages (Fig. 5). Thus, these data indicate that reduction of infection by macrophages cultured in hypoxia is no related to these cytokines. IL-6, among the cytokines evaluated in the present study, was the only one that was produced in higher quantity by infected macrophages in hypoxia than in normoxia (Fig 5B). Previous studies have shown IL-6 can inhibit leishmanicidal effect of TNF-α in cultured macrophages (66) and can inhibit the activation of macrophages by IFN-γ and TNF-α and subsequent killing of *L. amazonensis* (67). Our data demonstrated that despite higher IL-6 production by infected macrophages in hypoxia, this cytokine cannot inhibit the antileishmanial effect of this microenvironment.

Various studies have evaluated the effect of hypoxia on phagocytic activity of macrophages. Alveolar macrophages showed reduced retention of red blood cells (6); peritoneal macrophages reduced phagocytosis of zymozan particles and latex beads under hypoxia (12); and reperfusion after hypoxia caused a 3-fold induction in colloidal carbon uptake by Kupffer cells (68). In contrast, Turner et al. (10) did not observe the influence of hypoxia on human monocyte cell line phagocytosis of opsonized red blood cells, and Matsumoto et al. (69) showed that there was no difference in binding and uptake of low-density lipoprotein (LDL) in human monocyte-derived macrophages. Moreover, Acosta-Iborra et al. (70) and Anand et al. (71) observed that peritoneal macrophages cultured in hypoxia showed increase in the phagocytosis of SRBC and latex beads as compared to normoxia condition. The reasons for the different results might be attributed to the array of different macrophage types tested, the extent and duration of hypoxia applied, and the nature of the particles used in the various studies. In the present study, we have demonstrated that hypoxia inhibits the phagocytosis of inert particles or fixed parasite (Fig 6A). Nevertheless, it appears that hypoxia did not affect living *L. amazonensis* entry into macrophages (Fig. 6B and C). We propose that in this cell system hypoxia did not induce marked changes in ligands, receptors, or cytoskeletal proteins of either macrophage or parasite that could have an effect on the uptake of *L. amazonensis* by macrophages. The parasite internalization is more complex than the internalization of inert particles or fixed parasites, since this process involves the action of macrophages and of the parasite, as previously demonstrated (72). An alternative hypothesis to explain the low infection in hypoxic condition would be parasite exocytosis by macrophages, a process involved in the liberation of amastigotes by macrophages (47). Moreover, alveolar macrophages in hypoxia presented red blood cells on their surface after phagocytosis in normoxia, suggesting exocytosis was induced by hypoxia (6). Our data show hypoxic treatment does not also induce the exocytic process of internalized particles by

macrophages (Fig 6D). Thus, *L. amazonensis* amastigotes may not be exocytized when macrophages are cultured in hypoxia. With these results we conclude that the phagocytosis and exocytosis are not related to the anti-*Leishmania* activity of macrophages under hypoxia.

Cells need a constant supply of oxygen and nutrients to produce energy in the form of ATP. An insufficient supply of oxygen and nutrients results in ATP depletion, which leads to impaired cell function and cell death (73). Macrophages are one of the cells which adapt to hypoxia by switching from an aerobic to an anaerobic glycolytic pathway for ATP production (74). However, anaerobic glycolysis is inefficient, consuming 15 times more glucose per ATP molecule produced than oxidative phosphorylation (73). This led us to analyze ATP production by macrophages to determine whether hypoxia induces energetic metabolism damage in macrophages, which could be responsible for reduced infection of macrophages by *L. amazonensis*. Macrophages cultured in mild hypoxia (6% O_2) showed similar levels of ATP to that in normoxic conditions (Fig. 7A). This result demonstrates that mild hypoxia cannot affect energetic metabolism of macrophages, which does not contribute to *L. amazonensis* death in hypoxic macrophage cultures. The depletion of ATP occurred only in macrophages cultured in severe hypoxia (<1% O_2) (Fig. 7B), as previously demonstrated (6,74,75). However, infected macrophages produce similar levels of ATP when cultured in severe hypoxia or normoxia (Fig. 7B). The similar levels of ATP in normoxia and hypoxia by infected macrophages indicate that the reduction of infection of macrophages in hypoxia is not related to the impairment of energetic metabolism of macrophages.

The survivor of *Leishmania* amastigotes inside macrophages is important for pathogenesis of disease (76). The parasite *Leishmania* is susceptible to chemicals produced by host cell, such as NO (48), or to drugs treatment such as miltefosine (77) or antimonials (76). In all of these cases, the apoptosis-like death was reported in *Leishmania* intracellular amastigotes. In order to

clarify the mode of action of hypoxia against *L. amazonensis*, we have investigated whether apoptosis is induced in intracellular amastigotes by this microenvironment. In contrast to IFN-γ+LPS-activated macrophages, that produce NO and induce apoptosis-like death in intracellular amastigotes (Fig. 8A; 48), infected macrophages under hypoxia have not shown intracellular amastigotes with DNA fragmentation in 24 or 48 h of culture (Fig. 8). Since the TUNEL assay is specific for DNA fragmentation induced by apoptosis, we conclude that apoptosis-like death does not occur in intracellular parasites in hypoxic conditions. Further studies may be conducted to address the question whether alternative pathways of cell death of intracellular parasites can be induced by hypoxic condition.

Recently, our group showed that hypoxia-inducible factor-1α (HIF-1α) can be activated in macrophages infected with *L. amazonensis* in an *in vitro* model (17) and is also expressed in mouse cutaneous lesions during infection with *L. amazonensis* (15). HIF is a heterodimeric transcriptional protein consisting of HIF-1α and HIF-1β components (78,79). Both HIF-1α and HIF-1β belong to the basic helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) family of transcription factors (80). Whereas HIF-1β is constitutively expressed in all cells, HIF-1α has been shown to stabilize and accumulate in cells during hypoxia, mainly through inhibition of its degradation by the ubiquitin–proteasome system (79,81). The expression of different genes is controlled at a transcriptional level by HIF-1α, including erythropoietin, vascular endothelial growth factor, glucose transporters and glycolytic enzymes (79). In addition to HIF-1α, another mammalian bHLH-PAS protein, HIF-2α, has been implicated in executing the hypoxia response (80). Structurally, HIF-2α and HIF-1α are closely related, sharing a high degree of homology in their amino acid sequence (82-84) and functional similarity regarding to hypoxic stabilization and binding to HIF-1β (85). In this report, the expression of HIF-2α in macrophages infected with *L. amazonensis* was analyzed. The present data revealed that HIF-2α immunoreactivity, similarly to

HIF-1α, is elevated in nuclei of macrophage infected with *L. amazonensis* (Fig. 9; 17). Using pimonidazole as a surrogate marker of hypoxia (86), that creates adducts with thiol-containing proteins in hypoxic cells (87-90), our previous work has demonstrated that *L. amazonensis*-infected macrophages under normoxia are not hypoxic cells (17), since pimonidazole adducts were barely detectable in these cultures. Then, the HIF-2α expression may be independent of hypoxia and a nonhypoxic stimulus must be involved in *L. amazonensis* activation of HIF-2α. Despite the pathway of HIF-1α and HIF-2α activation is not known during *L. amazonensis* infection, the induction of these proteins and the target genes constitute part of an adaptative mechanism resulting from parasite infection and that this could permit the host cell to attenuate damage, maintain integrity and survive such infection, as discussed in Degrossoli et al. (17).

To summarize, we have shown that hypoxia induces macrophage resistance to *L. amazonensis* and we investigated possible mechanisms that are involved in this phenomenon. We show hypoxia acts on macrophages altering ROS and cytokines production, phagocytic processes and energetic metabolism. However, with exception of ROS, these parameters are not altered in *L. amazonensis*-infected macrophages by reduced oxygen tension. Although the data presented here do not explain the exact mechanism by which macrophages control *L. amazonensis* infection in hypoxia, we cannot exclude the participation of ROS in the anti-*Leishmania* activity of macrophages in hypoxia. Furthermore, these results support the notion that hypoxia, a microenvironmental factor present in diseased tissues, modulates macrophage protein expression and functional activity, and the notion that the effects of hypoxia on macrophages observed *in vitro* may play a role in tissue damage occurring during the course of leishmaniasis.

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LEGENDS

Figure 1. Western blot analysis of iNOS in J774 macrophages. Macrophages exposed to IFN-γ+LPS (lane 1), cultured in normoxia for 24 h (lane 2), infected with *L. amazonensis* amastigotes and cultured in normoxia for 24 h (lane 3), cultured in hypoxia for 24 h (lane 4), and infected with *L. amazonensis* and cultured in hypoxia for 24 h (lane 5). iNOS was detected by Western blot as described in Materials and Methods.

Figure 2. Effect of hypoxia on *L. amazonensis* infected-macrophages. Peritoneal wild type and iNOS knockout macrophages were infected with *L. amazonensis* amastigotes and cultured in normoxia or hypoxia for 24 h. The percentage of infected macrophages (A) and the number of amastigotes per macrophage (B) were determined as described in Materials and Methods. The result represents the mean ± SD of 3 experiments. The significance of the difference between cells cultures in normoxia and hypoxia is indicated: * $P \leq 0{,}05$.

Figure 3. Effect of hypoxia on ROS production by macrophages. Peritoneal macrophages were treated with PMA or infected with *L. amazonensis* amastigotes and incubated for 1.5 h (A) or 3 h (B) under normoxia or hypoxia. ROS production was determined in the culture by DCF probe as described in Materials and Methods. The results represent the mean ± SD of 3 experiments. The significance of the difference between cell cultures in hypoxia and normoxia is indicated in the figure. *$P \leq 0.05$. The significance of the difference between treated and control cell cultures is indicated in the figure. #$P \leq 0.05$.

Figure 4. Effect of antioxidants on *L. amazonensis* infected-macrophages. Peritoneal macrophages were infected with *L. amazonensis* amastigotes and cultured in normoxia for 1 h. Extracellular amastigotes were removed and the cultures were maintained in normoxia or hypoxia with antioxidants NAC or Ebselen for 24 h, as described in Materials and Methods. The percentage of infected macrophages treated with NAC (A), the number of amastigotes per macrophage treated with NAC (B), the percentage of infected macrophages treated with Ebselen (C), and the number of amastigotes per macrophage treated with Ebselen (D) were determined as described in Materials and Methods. The result represents the mean ± SD of 3 experiments. The

significance of the difference between cells cultures in normoxia and hypoxia is indicated: * $P \leq$ 0,05.

Figure 5. Effect of hypoxia on cytokines release by macrophages. Peritoneal macrophages were treated with LPS or IFN-γ+LPS or infected with *L. amazonensis* amastigotes and incubated for 24 h under normoxia or hypoxia. TNF-α activity was determined in the culture supernatants by L929 citotoxicity assay and IL-6, IL-12 and IL-10 production was determined in the culture supernatants by ELISA assay. The results represent the mean ± SD of 3 experiments. The significance of the difference between cell cultures in hypoxia and normoxia is indicated in the figure. *$P \leq 0.05$. The significance of the difference between treated and control cell cultures is indicated in the figure. #$P \leq 0.05$.

Figure 6. Effect of hypoxia on phagocytosis and exocytosis by macrophages. (A) Peritoneal macrophages were incubated with FITC-labeled *Escherichia coli* K-12 bioparticles for 1 h under normoxia or hypoxia. Alternatively, macrophages were incubated with carboxylate-modified microspheres pre-treated with PBS (control), BSA or immunized serum (opsonized) for 1 h in normoxia or hypoxia. Intracellular particles were quantified by measuring the fluorescence intensity of the cells using a fluorescence spectrometer microplate reader, as described in Materials and Methods. (B and C) Peritoneal macrophages were incubated with living or glutaraldehyde-fixed *L. amazonensis* amastigotes and cultured in normoxia or hypoxia for 1 h. Extracellular amastigotes were removed and the percentage of infected macrophages (B) and the number of amastigotes per macrophage (C) were determined as described in Materials and Methods. (D) Peritoneal macrophages were incubated with FITC-labeled *Escherichia coli* K-12 bioparticles for 1 h under normoxia. Thus, macrophages were washed to remove extracellular particles and were further incubated for 1 h and 24 h in normoxia or hypoxia. Intracellular particles were quantified by measuring the fluorescence intensity of the cells. The fluorescence of each condition was normalized to the fluorescence of macrophages in normoxia at initial time (0 h). The result represents the mean ± SD of 3 experiments. The significance of the difference between cells cultures in normoxia and hypoxia is indicated: * $P \leq 0.05$. The significance of the difference between treated and control cell cultures is indicated in the figure. #$P \leq 0.05$.

Figure 7. Effect of hypoxia on ATP release by *L. amazonensis*-infected macrophages. Peritoneal macrophages were infected with *L. amazonensis* amastigotes and incubated for 24 h under normoxia (21% O_2) or under hypoxia with 6% O_2 (A) or <1% O_2 (B). ATP concentrations were determined in the culture supernatants by the bioluminescence assay as described in Materials and Methods. The results represent the mean ± SD of 3 experiments. The significance of the difference between cell cultures in hypoxia and normoxia is indicated in the figure. $*P \leq 0.05$.

Figure 8. In situ analysis (TUNEL staining) of apoptosis in *L. amazonensis*-infected macrophages. Peritoneal macrophages were infected with *L. amazonensis* amastigotes and incubated under normoxia or hypoxia, for 24 h (Panel A) or 48 h (Panel B) or stimulated with IFN-γ+LPS in normoxia for 24 h (panel A). DNA fragmentation analysis was determined by the TUNEL method and analyzed under a fluorescent and phase contrast microscope (bars = 12 µm). Cell nuclei were stained with DAPI and merged with phase contrast. *L. amazonensis*-harboring parasitophorous vacuoles are indicated by arrowheads.

Figure 9. Expression of HIF-2α by peritoneal macrophages. The cells were infected with *L. amazonensis* amastigotes or left uninfected (Control) and exposed to normoxia or hypoxia for 24 h, as described in Materials and Methods. The cells were fixed and stained with anti-HIF-2α antibody. Isotype and secondary antibody controls were negative for staining (data not shown). Insets show cell nuclei stained with DAPI (bars = 20 µm). *L. amazonensis*-harboring parasitophorous vacuoles are marked by asterisks

TABLE

Table 1. Effect of hypoxia on nitrite production by macrophages infected with *Leishmania amazonensis* and activated with IFN-γ+LPS.

Nitrite concentrations were determined in the culture supernatants by the Griess reaction as described in Materials and Methods. Nitrite concentration in µM.

	Normoxia	*Hypoxia*
Control	N.D[a].	N.D.
Infected[b]	N.D.	N.D.
IFN-+LPS[c]	12,05±0,67[*]	-

[a] N.D.: not detected.

[b]J774 macrophages infected with *L. amazonensis* amastigotes for 24 h.

[c]J774 macrophages treated with IFN-γ+LPS during 8 h.

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 1

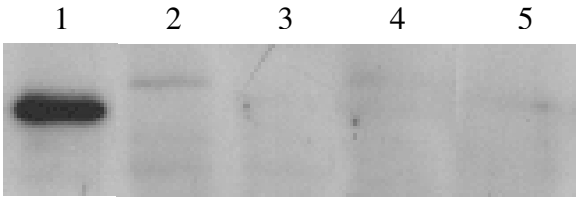


Figure 2

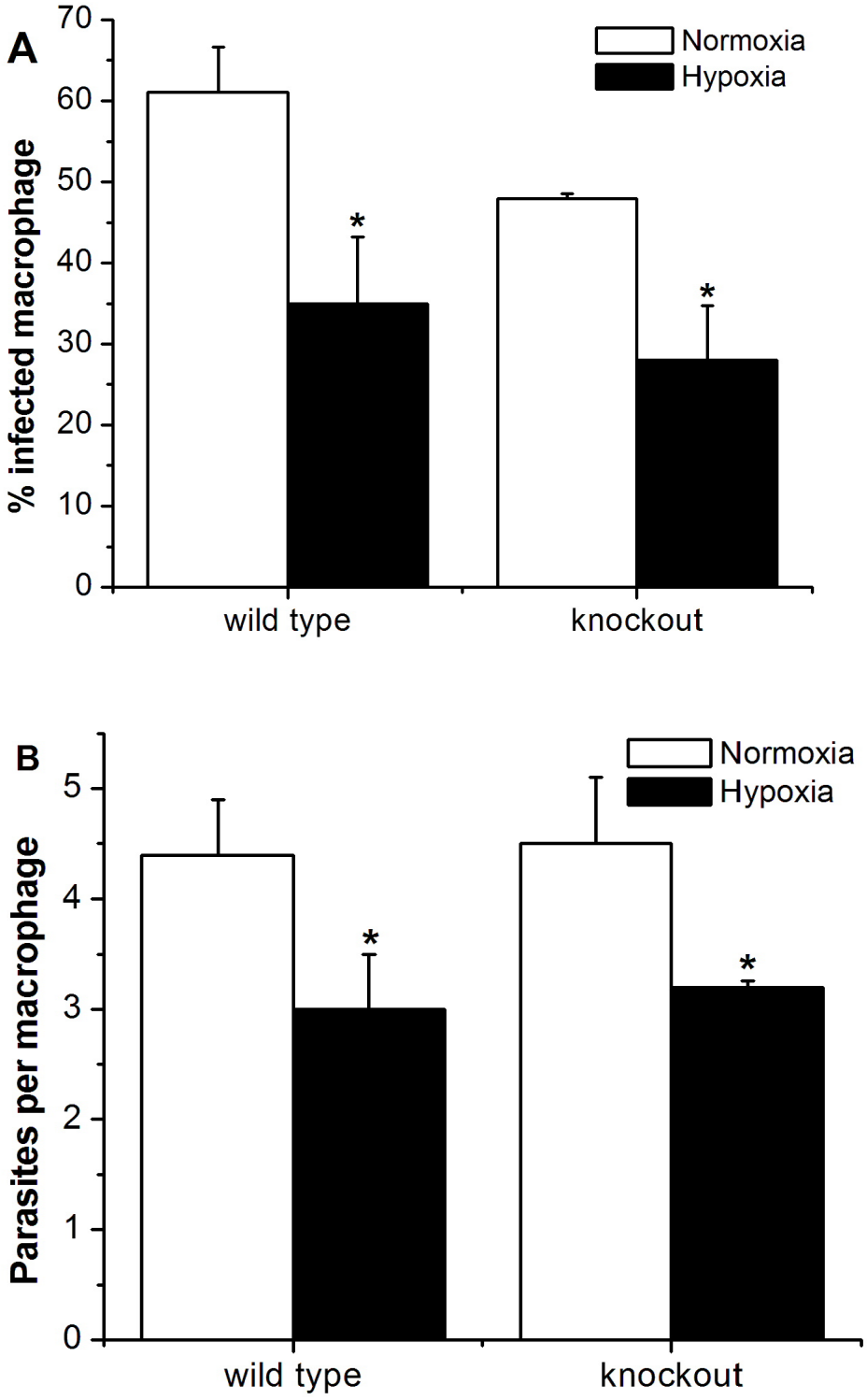

A
% infected macrophage
70
60
50
40
30
20
10
0
Normoxia
Hypoxia
*
*
wild type
knockout
B
Parasites per macrophage
5
4
3
2
1
0
Normoxia
Hypoxia
*
*
wild type
knockout

Figure 3

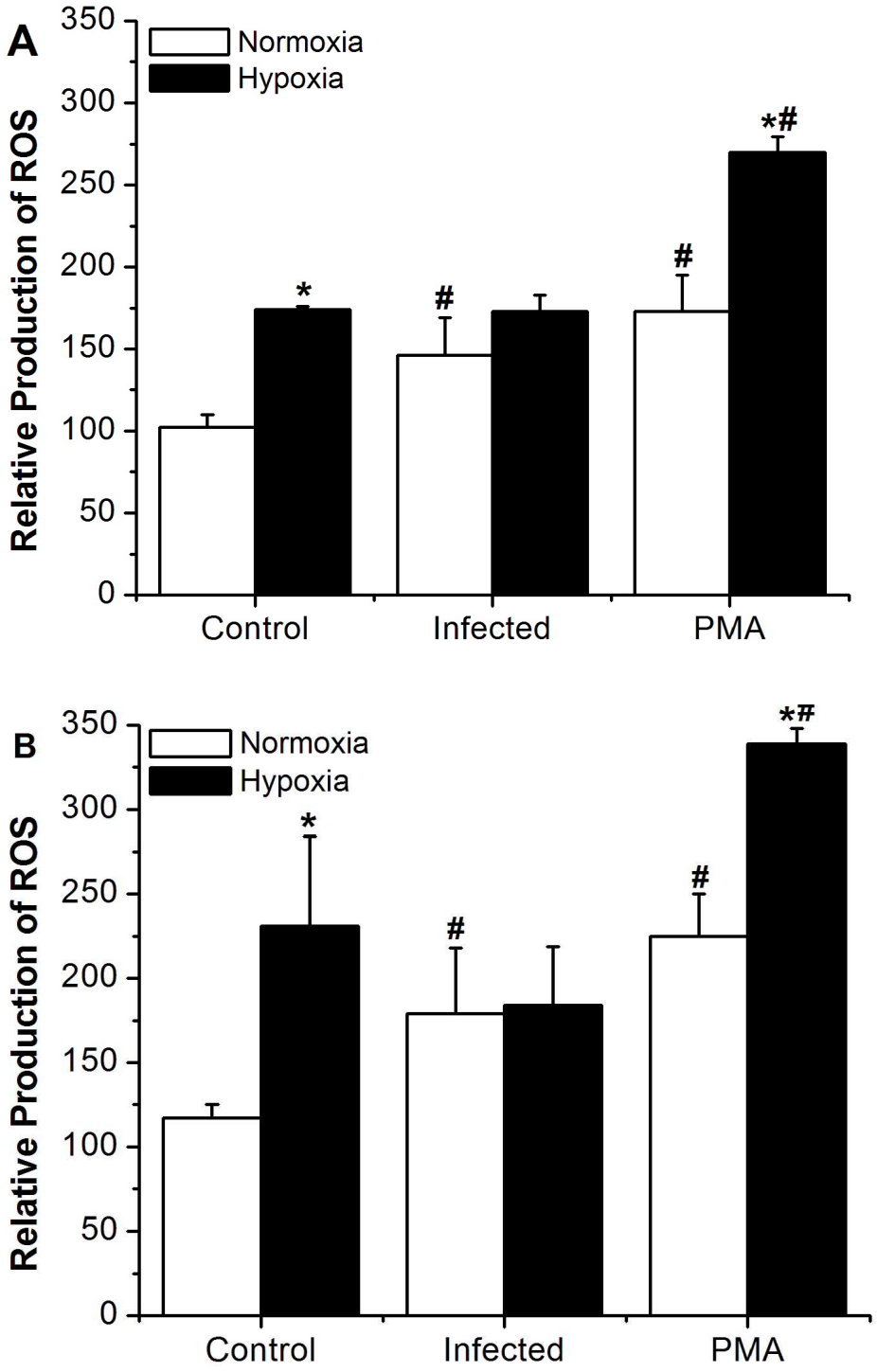

A
Normoxia
Hypoxia
Relative Production of ROS
350
300
250
200
150
100
50
0
*
#
#
*#
Control
Infected
PMA
B
Normoxia
Hypoxia
Relative Production of ROS
350
300
250
200
150
100
50
0
*
#
#
*#
Control
Infected
PMA

Figure 4

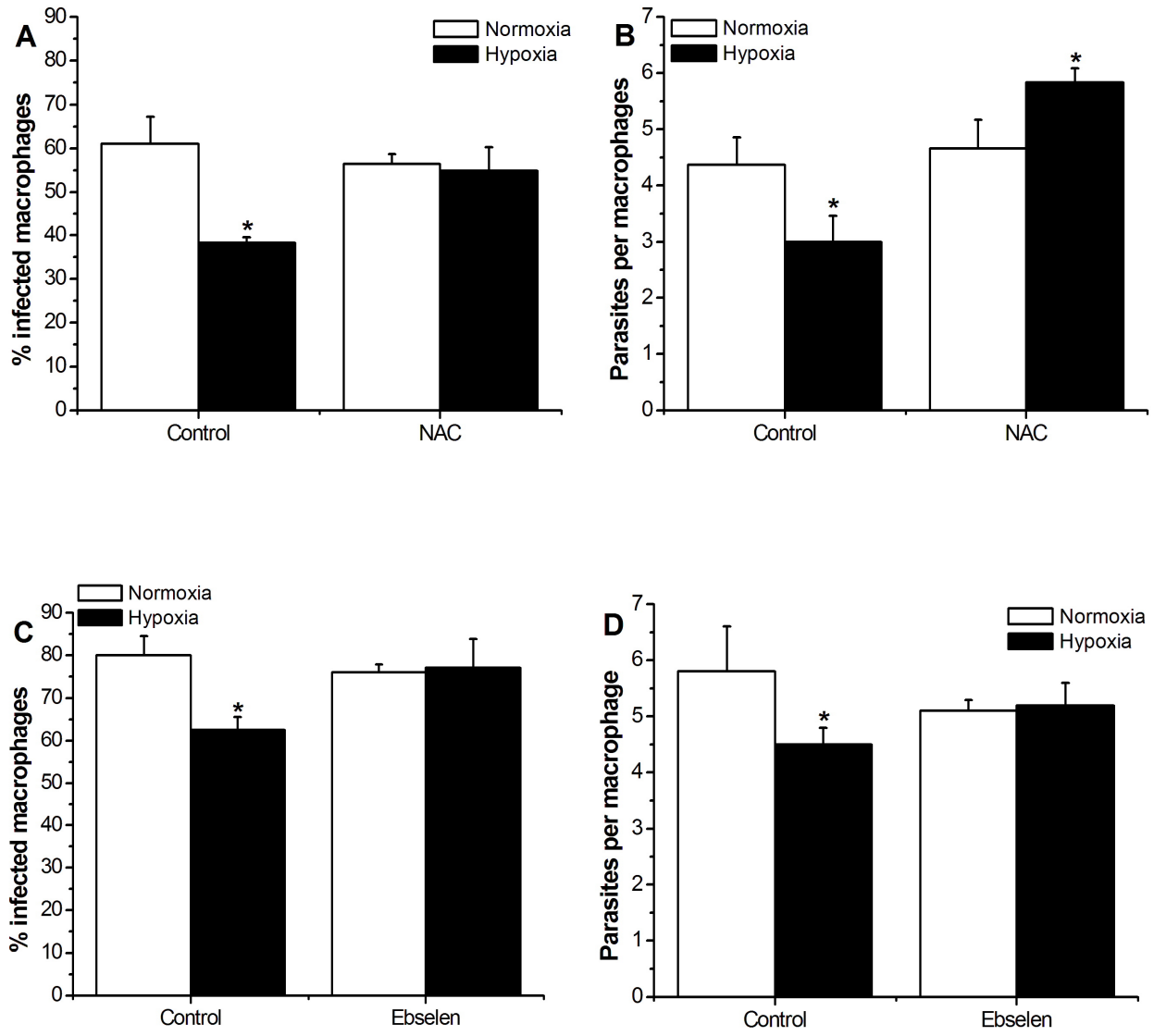

A
% infected macrophages
Normoxia
Hypoxia
Control
NAC
B
Parasites per macrophages
Normoxia
Hypoxia
Control
NAC
C
% infected macrophages
Normoxia
Hypoxia
Control
Ebselen
D
Parasites per macrophage
Normoxia
Hypoxia
Control
Ebselen

Figure 5

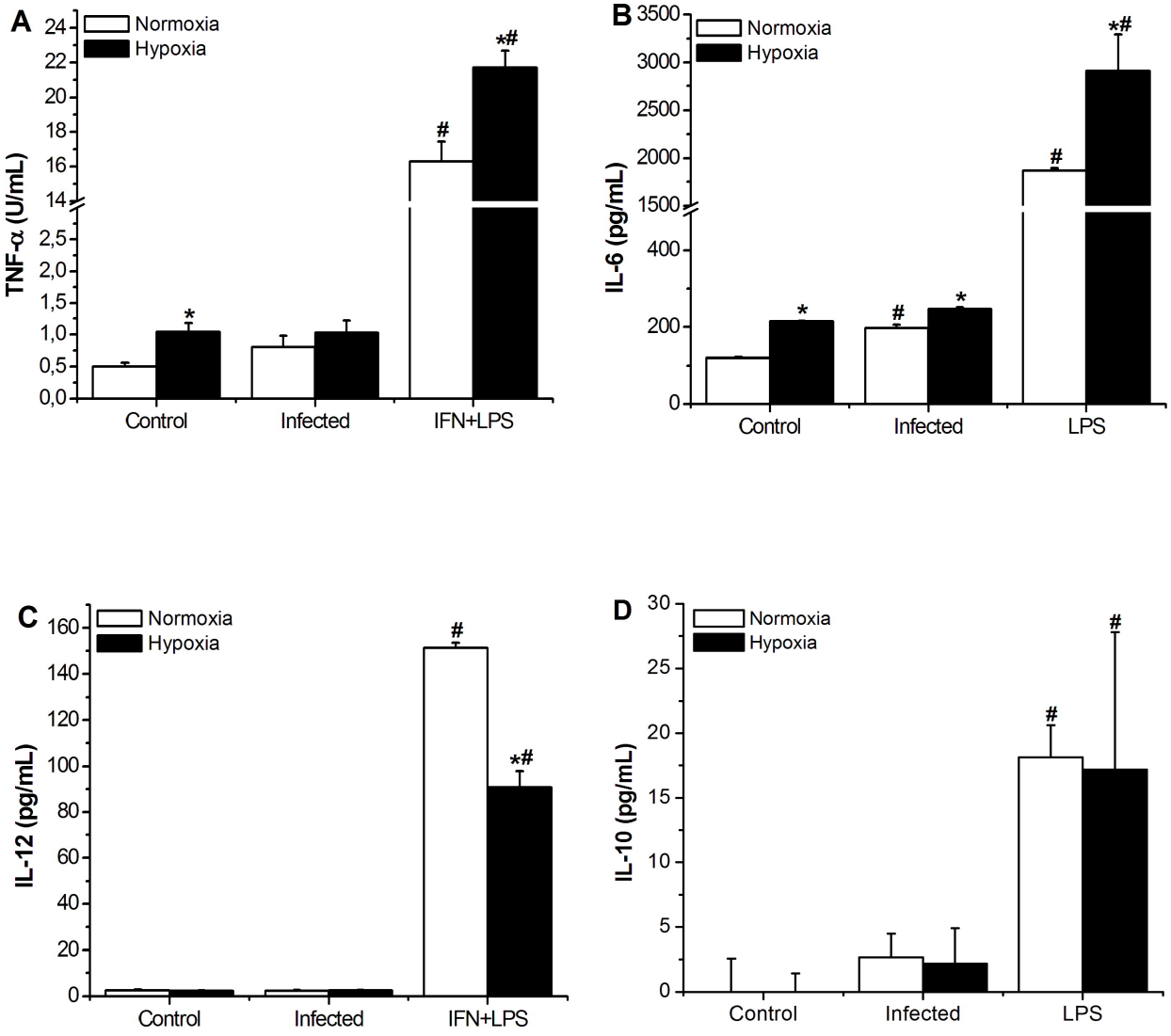

A
Normoxia
Hypoxia
TNF-α (U/mL)
Control
Infected
IFN+LPS
B
Normoxia
Hypoxia
IL-6 (pg/mL)
Control
Infected
LPS
C
Normoxia
Hypoxia
IL-12 (pg/mL)
Control
Infected
IFN+LPS
D
Normoxia
Hypoxia
IL-10 (pg/mL)
Control
Infected
LPS

Figure 6

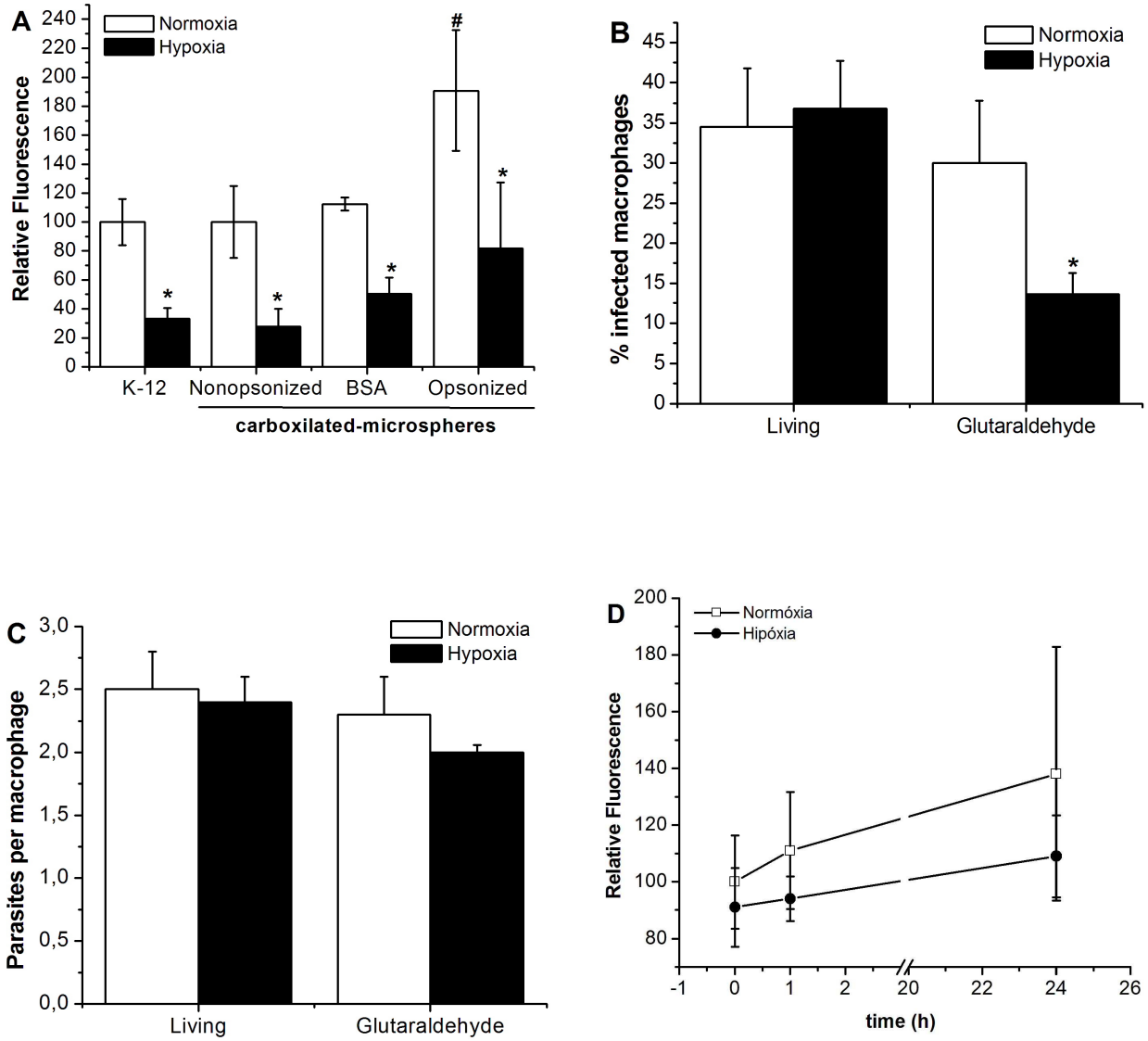

A
Normoxia
Hypoxia
Relative Fluorescence
K-12
Nonopsonized
BSA
Opsonized
carboxilated-microspheres
B
Normoxia
Hypoxia
% infected macrophages
Living
Glutaraldehyde
C
Normoxia
Hypoxia
Parasites per macrophage
Living
Glutaraldehyde
D
Normóxia
Hipóxia
Relative Fluorescence
time (h)

Figure 7

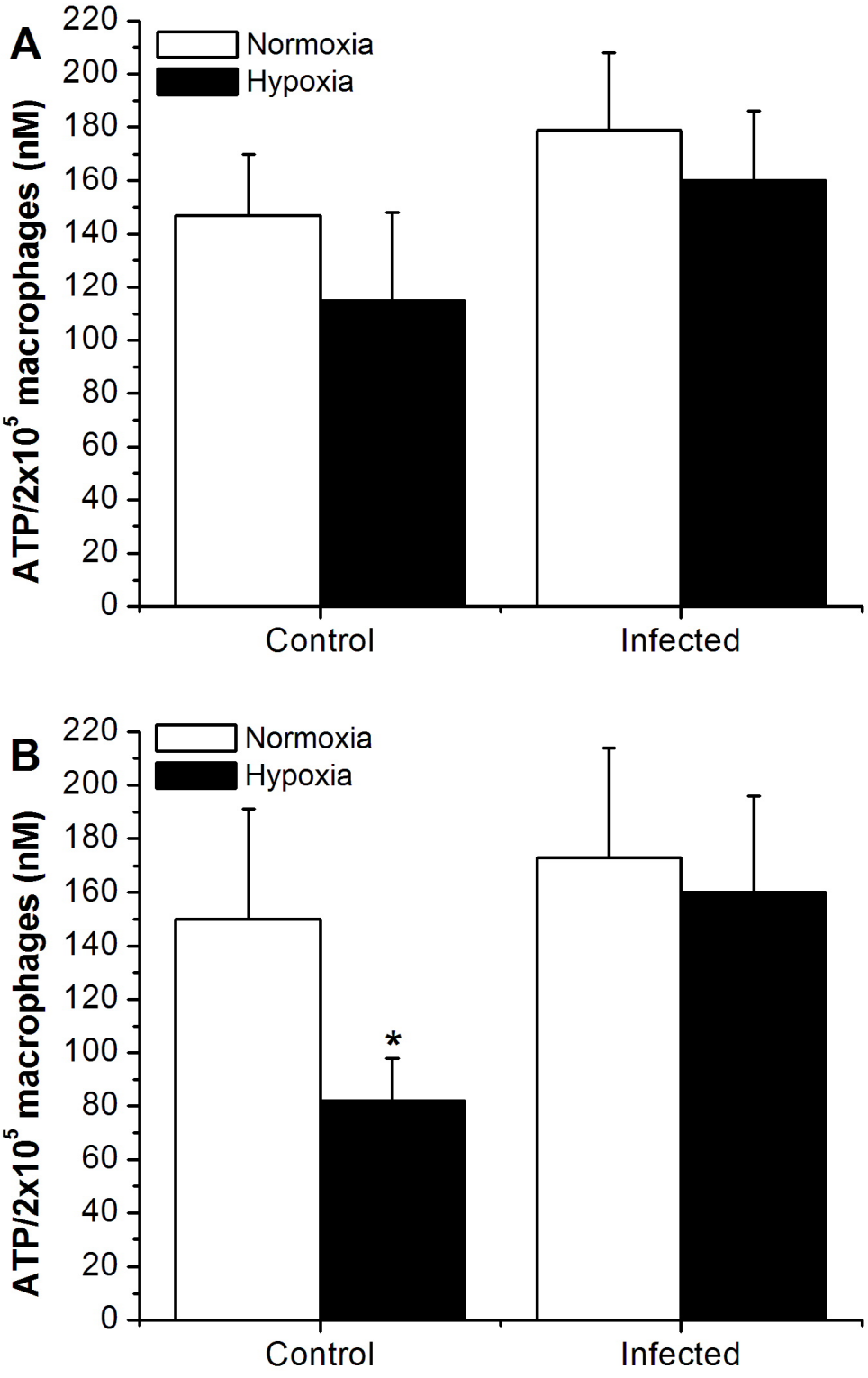

A
Normoxia
Hypoxia
ATP/2x10^5 macrophages (nM)
220
200
180
160
140
120
100
80
60
40
20
0
Control
Infected
B
Normoxia
Hypoxia
ATP/2x10^5 macrophages (nM)
*
220
200
180
160
140
120
100
80
60
40
20
0
Control
Infected

Figure 8

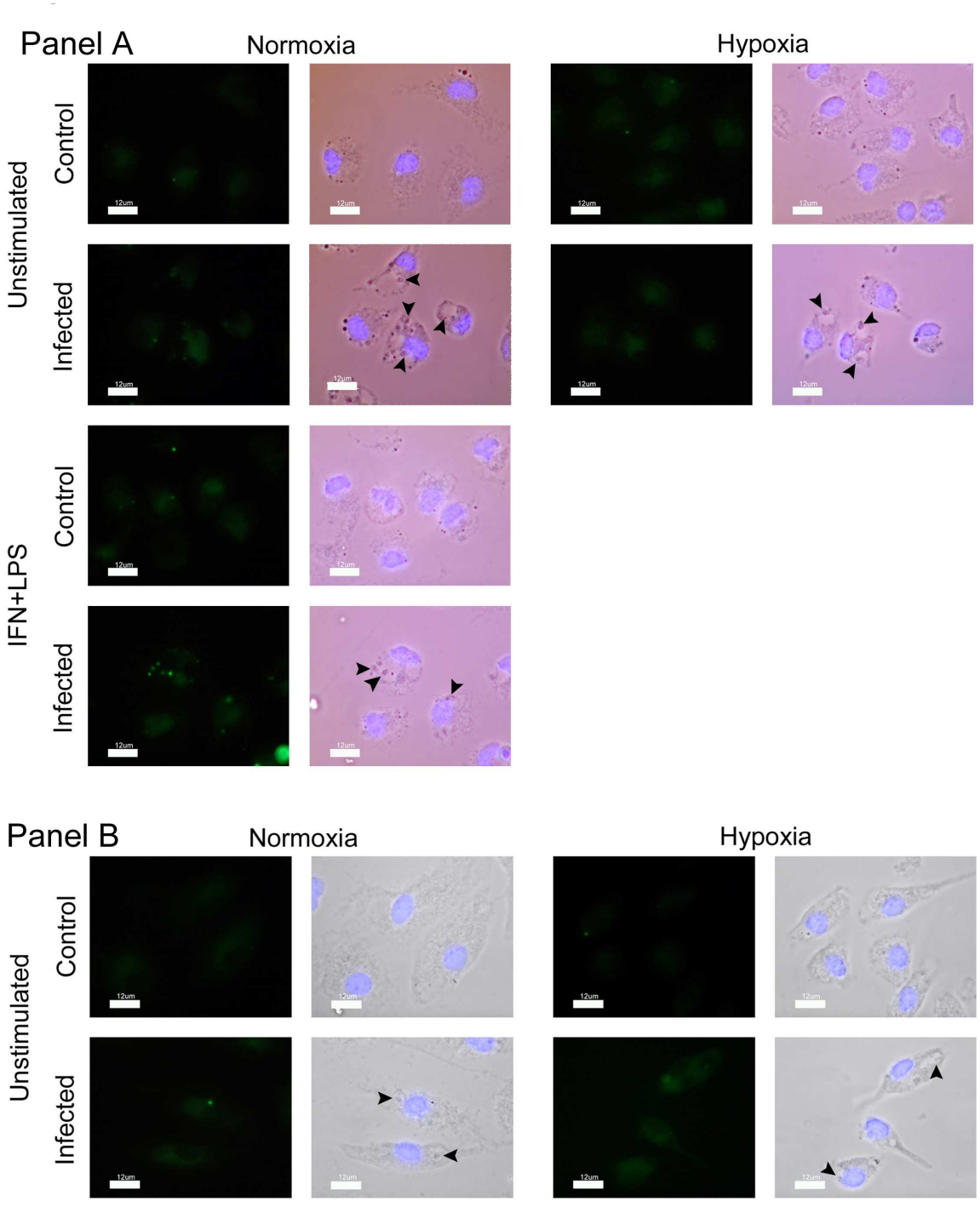
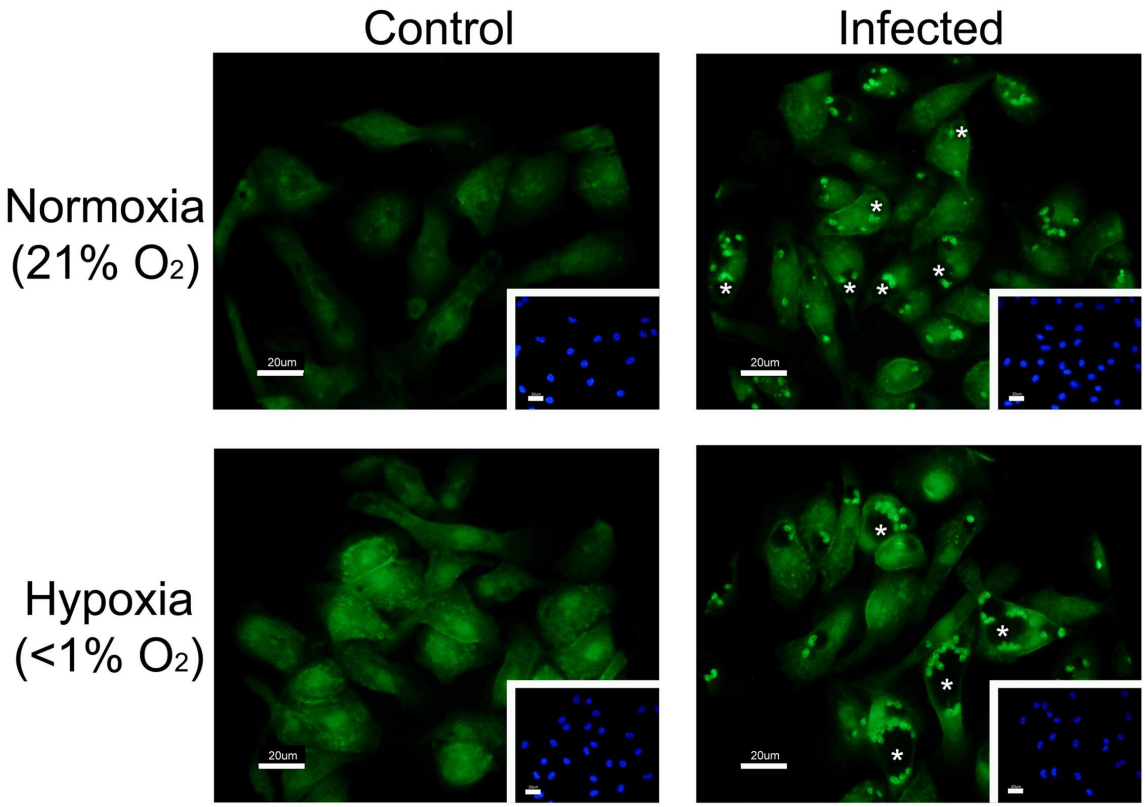

Panel A
Normoxia
Hypoxia
Unstimulated
Control
Infected
IFN+LPS
Control
Infected
Panel B
Normoxia
Hypoxia
Unstimulated
Control
Infected

Figure 9


Control
Infected
Normoxia
(21% O2)
Hypoxia
(<1% O2)
20um
20um
20um
20um

4.2. PROLIFERAÇÃO DE LINFÓCITOS E APRESENTAÇÃO DE ANTÍGENOS POR MACRÓFAGOS EM HIPÓXIA

Macrófagos desempenham importantes papéis nas respostas imunes inata e adaptativa através das funções específicas de fagocitose, apresentação de antígeno, secreção de citocinas e remodelamento tecidual. Como fagócitos profissionais os macrófagos expressam uma variedade de receptores que participam da internalização fagocítica de antígenos. Quando ativados, macrófagos são induzidos a produzirem citocinas como IL-12, IL-18, IFN-γ, TNF-α e IL-1β (DI MARZIO et al., 1994; FRUCHT et al., 2001; FULTZ et al., 1993; LE PAGE et al., 2000; MUNDER et al., 1998; MURATA et al., 2000; PUDDU et al., 1997; SCHINDLER et al., 2001). Estas citocinas, além de direcionar uma resposta inflamatória pelo aumento da capacidade fagocítica dos macrófagos, promovem o processamento e apresentação de antígeno pelos macrófagos e a ativação e diferenciação de células Th1 secretoras de IFN-γ, células T citotóxicas (CTL) e células *natural killers* (NK) (DOODY et al., 2007; GIROUX; SCHMIDT; DESCOTEAUX, 2003; HARING; CORBIN; HARTY, 2005). O objetivo da apresentação de antígeno é amplificar o sinal através da produção de citocinas por células T e expansão clonal. Macrófagos são células envolvidas em várias doenças inflamatórias tais como placas ateroscleróticas, infarto do miocárdio, artrite reumatóide, cicatrização, infecções bacterianas e tumores malignos em que áreas de hipóxia estão presentes (LEWIS et al., 1999; MURDOCH; LEWIS, 2005).

Poucos trabalhos na literatura avaliaram a função "apresentadora de antígeno" (APC) de macrófagos durante processos hipóxicos. Murata et al. (2002) e Lahat et al. (2003) demonstraram que macrófagos e células dendríticas murinas produzem grandes quantidades de interferon gama sob condições hipóxicas (MURATA et al., 2002) e que a expressão de CD80 (molécula co-

estimulatória de superfície, envolvida na apresentação de antígenos) está reduzida em monócitos humanos cultivados em hipóxia (LAHAT et al., 2003). Recentemente foi relatado que macrófagos em hipóxia aumentam a expressão de moléculas co-estimulatórias e moléculas envolvidas na apresentação de antígeno, ativando mais eficientemente células T na presença de um antígeno específico (ACOSTA-IBORRA et al., 2009). Um dos objetivos deste trabalho de tese foi avaliar a capacidade APC de macrófagos em hipóxia. Abordamos essa questão utilizando o modelo da leishmaniose, isto é, pulsando macrófagos com antígenos totais de *L. amazonensis* em hipóxia e verificando a capacidade proliferativa de linfócitos T (específicos, de animais imunizados).

METODOLOGIA

1. Células

Os macrófagos peritoneais foram obtidos através do lavado intraperitoneal de camundongos isogênicos BALB/c fêmeas, de oito a 12 semanas de idade e Specific Pathogen Free (SPF), fornecidos pelo Centro Multidisciplinar para Investigação Biológica (CEMIB)/UNICAMP. Os camundongos foram sacrificados e 10 mL de solução salina estéril foram injetados no peritôneo do animal. A pele foi rebatida e, com auxílio de seringa, o exsudato retirado, armazenando-o em frasco imerso em banho de gelo. O número de células foi contado em câmara de Neubauer, considerando-se que 50% das células são constituídas de macrófagos (BARBIÉRI et al., 1993). As células foram então distribuídas, na concentração de 10^5 macrófagos/0,2 mL, em placas de cultura de 96 poços, incubando-se por 2 h a temperatura ambiente, para adesão dos macrófagos. Após esse período, o exsudato foi substituído por meio

RPMI 1640 suplementado com 25 μg/mL de Gentamicina e 10% de soro fetal bovino. A cultura foi mantida em estufa incubadora úmida (5% CO_2, 95% Ar) a 37°C.

2. Parasita

O parasita *Leishmania (Leishmania) amazonensis* (MHOM/BR/M2269) é mantido no laboratório nas formas amastigota e promastigota. Os amastigotas são mantidos em camundongos BALB/c (linhagem susceptível à infecção), que são infectados subcutaneamente no coxim plantar de uma das patas traseiras (GIORGIO et al., 1998). Para a utilização do parasita nos ensaios *in vitro*, as formas amastigotas foram retiradas das lesões cutâneas e purificadas segundo Barbiéri et al. (1993).

3. Ambiente hipóxico

Para os experimentos em hipóxia foi utilizada uma mistura padrão de gases ("mistura hipóxica") contendo 2% de O_2, 5% de CO_2 e Nitrogênio para balancear a tensão dos gases ou apenas 5% CO_2 e Nitrogênio (White Martins S/A – Campinas/SP). As placas de cultura contendo as células dispostas foram expostas ao microambiente hipóxico utilizando-se uma câmara hipóxica (Billups-Rothenberg). As culturas celulares foram colocadas na câmara hipóxica, a qual foi preenchida imediatamente com a "mistura hipóxica" durante 15 min, com uma vazão de 2 L/min. Em seguida, as mangueiras de entrada e saída de gás foram fechadas e a câmara mantida em estufa incubadora a 37°C. Nestas condições, a tensão de oxigênio no meio de cultura foi 35 mmHg (6% O_2). Em meio normóxico, a pO_2 é 150 mmHg (21% O_2) (COLHONE et al., 2004; DEGROSSOLI et al., 2004).

4. Infecção de macrófagos com *L. amazonensis*

Os macrófagos peritoneais foram cultivados em placas de cultura de 96 poços, na concentração de 10^5 macrófagos/0,2 mL/poço. Estas células foram infectadas com amastigotas de

L. amazonensis na proporção 3:1 parasitas:macrófago e mantidos em normóxia a 37º C por 1 h. Após esse período, os macrófagos foram lavados, retirando os parasitas extracelulares, e receberam meio fresco. As culturas foram então incubadas em normóxia por 24 h a 37°C (COLHONE et al., 2004) e, em seguida, foram utilizadas nos ensaios de apresentação de antígeno (item 6).

5. Ensaio de proliferação

As células do linfonodo (LNC) foram coletadas de camundongos imunizados com antígeno de *L. amazonensis* s.c., 12 dias após a imunização. A suspensão de LNC foi preparada em meio RPMI completo contendo 5% SFB. Essas células foram cultivadas em quintuplicatas em placas de 96 poços a 5×10^5 células/poço na presença de diferentes concentrações de antígeno de *L. amazonensis*: equivalente a 5 ou 20×10^6 parasitas/mL. As culturas foram incubadas por 96 h a 37°C e 5% CO_2, em normóxia ou hipóxia (6% O_2). Alternativamente, as células do linfonodo foram cultivadas na presença de 2,5 μg/mL de Concanavalina A (ConA - Sigma) ou 10 μg/mL de LPS. Neste caso, as culturas foram incubadas por 72 h a 37°C e 5% CO_2, em normóxia ou hipóxia. Após o período de incubação, a taxa de proliferação dos linfócitos foi medida pelo ensaio do MTT (Sigma), de acordo com as instruções do fabricante.

6. Preparação de APC *in vitro* e ensaio de apresentação de antígeno

Macrófagos peritoneais foram utilizados como células apresentadoras de antígeno (APCs) nos ensaios de apresentação de antígeno. Macrófagos peritoneais foram coletados de camundongos BALB/c, como descritos no item 1, e foram distribuídos 10^5 macrófagos/poço da microplaca, contendo 200 μL de meio RPMI completo com 10% de SFB. Os macrófagos foram infectados com amastigotas de *L. amazonensis* na proporção 3:1 parasita:célula, conforme item 5.4, e mantidos a 37ºC, 5% CO_2 por 24 h. Após esse período, macrófagos de outros poços foram

pulsados com antígeno de *L. amazonensis* em diferentes concentração: 10, 20 ou 40 equivalente parasita/macrófago. Macrófagos infectados ou pulsados foram mantidos a 37°C e 5% CO_2 por 4 h, em normóxia ou hipóxia (6% O_2). Em seguida, os macrófagos foram co-cultivados com 10^6 LNC/poço por 96 h em normóxia, a 37°C e 5% CO_2. Após esse período, a taxa de proliferação dos linfócitos foi medida pelo ensaio do MTT (Sigma), de acordo com as instruções do fabricante.

7. Análise dos resultados

Todos os ensaios foram realizados em situações de normóxia, hipóxia, normóxia + infecção e hipóxia + infecção. Pelo menos três experimentos de cada um dos itens descritos acima foram realizados separadamente e os resultados são expressos pela média ± SD. Os valores foram comparados usando-se o "Student's *t*-test".

RESULTADOS

1. Proliferação de linfócitos

A resposta dos linfócitos de animais imunizados com antígenos de *L. amazonensis* foi avaliada através do ensaio de linfoproliferação em hipóxia e normóxia. Essa capacidade proliferativa foi medida através do ensaio do MTT, como descrito em Metodologia. As células dos linfonodos incubadas na presença de antígeno de *L. amazonensis* proliferaram em normóxia, enquanto as células cultivadas em meio de cultura sem antígeno (controle) não proliferaram (Fig. 1). Em ambiente hipóxico, as células dos linfonodos controle e as células cultivadas na presença de antígeno não proliferaram, semelhante as células controle em normóxia (Fig. 1). Assim, há proliferação dos linfócitos na presença do antígeno apenas em ambiente normóxico.

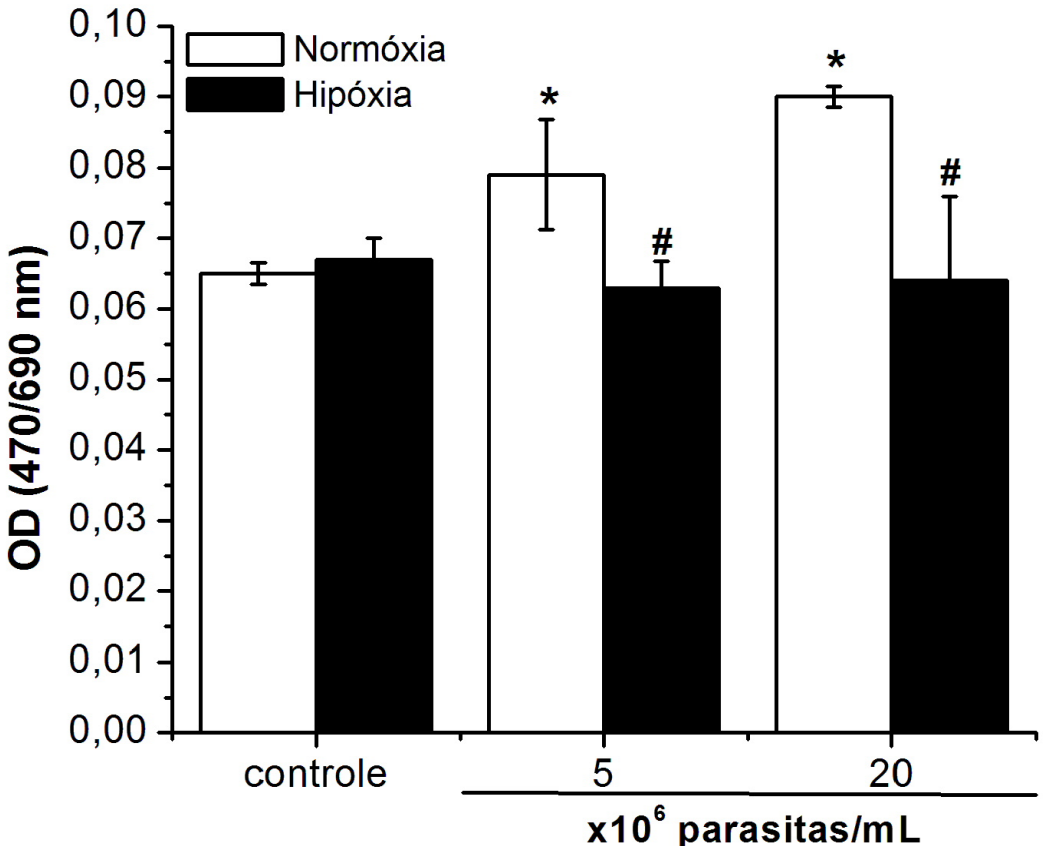


Figura 1. Proliferação de linfócitos. Células de linfonodos obtidas de camundongos imunizados, 12 dias após a imunização, foram estimuladas in vitro por 96 h de cultura, em normóxia ou hipóxia, com antígenos de *L. amazonensis* a diferentes concentrações: 5 ou 20 x 10^6 parasitas/mL. Alternativamente, as células dos linfonodos foram incubadas em meio sem antígeno (controle). A proliferação dos linfócitos foi quantificada pelo ensaio do MTT, conforme "Metodologia", em que a densidade ótica (OD) obtida é proporcional a quantidade de células. Os resultados representam média ± DP de um experimento realizado em quintuplicata. *P < 0.05 em relação a cultura de linfócitos controle em normóxia. #P<0,05 entre normóxia e hipóxia.

Para analisar se o ambiente hipóxico altera a capacidade de reconhecimento do antígeno pelos linfócitos ou a sua capacidade proliferativa, cultivamos as células dos linfonodos de camundongos imunizados na presença de Concanavalina A (ConA), um mitógeno específico de linfócitos T, ou na presença de LPS, um mitógeno de células B. Nesses ensaios, as células proliferam na presença dos mitógenos independente da apresentação do antígeno. Na Figura 2 observamos que as células dos linfonodos cultivadas com LPS ou ConA proliferaram. A proliferação dos linfócitos estimulados com ConA foi cerca de 15 vezes maior e a de células estimuladas com LPS foi cerca de duas vezes maior do que a proliferação de células não estimuladas (Fig. 2). Quando analisamos a proliferação dos linfócitos controle ou cultivados com LPS, observamos que não há diferença entre normóxia e hipóxia (Fig. 2). Células dos linfonodos estimuladas com ConA em ambiente hipóxico apresentem uma proliferação maior do que linfócitos controle neste mesmo ambiente. Entretanto, a capacidade de proliferação dos linfócitos na presença de ConA em hipóxia foi cerca de um terço da capacidade proliferativa dos linfócitos estimulados com ConA em normóxia (Fig. 2). Como houve diminuição da proliferação dos linfócitos T em hipóxia, concluímos que este microambiente altera sua capacidade proliferativa.

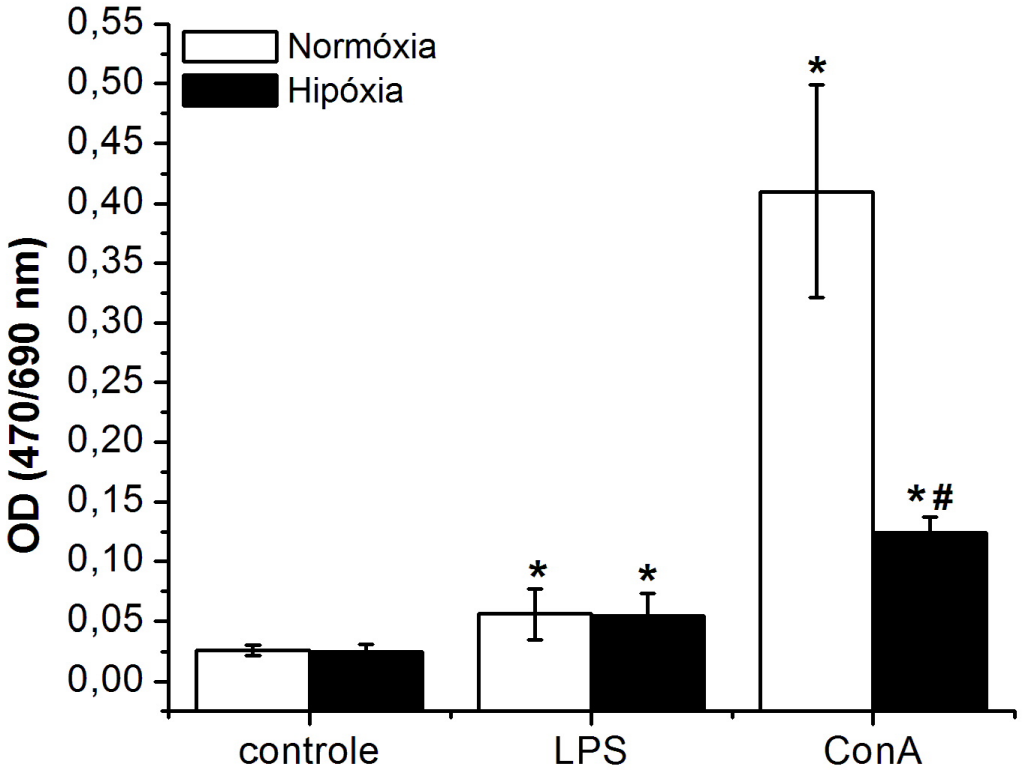


Figura 2. Proliferação de linfócitos. Células do linfonodo obtidas de camundongos imunizados, 12 dias após a imunização, foram estimuladas in vitro por 72 h de cultura, em normóxia ou hipóxia, com 10 µg/mL de LPS (LPS) ou 2,5 µg/mL de Concanavalina A (ConA). Alternativamente, as células dos linfonodos foram incubadas em meio sem mitógeno (controle). A proliferação dos linfócitos foi quantificada pelo ensaio do MTT, conforme "Metodologia", em que a densidade ótica (OD) obtida é proporcional a quantidade de células. Os resultados representam média ± DP de um experimento realizado em quintuplicata. *P < 0.05 em relação a cultura de linfócitos controle em normóxia. #P<0,05 entre normóxia e hipóxia.

2. Apresentação de antígenos por macrófagos

A capacidade de apresentação de antígeno (APC) dos macrófagos também foi avaliada. Estas células foram infectadas ou pulsadas com antígenos de *L. amazonensis* em normóxia ou hipóxia e, posteriormente, foram cultivadas com células de linfonodos de camundongos imunizados com antígeno de *L. amazonensis* em normóxia. As células do linfonodo cultivadas com macrófagos infectados ou pulsados proliferaram, enquanto linfócitos cultivados com macrófagos controle (não infectados ou não pulsados) não proliferaram (Fig. 3). As células de linfonodo cultivadas com macrófagos infectados com *L. amazonensis* proliferaram de modo semelhante em ambiente normóxico ou hipóxico (Fig. 3). Isto indica que a capacidade APC de macrófagos infectados com *L. amazonensis* não é alterada pela hipóxia. A proliferação dos linfócitos cultivados com macrófagos previamente pulsados com antígeno de *L. amazonensis* em hipóxia foi maior do que a proliferação de linfócitos cultivados com macrófagos pulsados em normóxia (Fig. 3). Este resultado indica que o ambiente hipóxico altera a capacidade de apresentação de antígeno dos macrófagos.

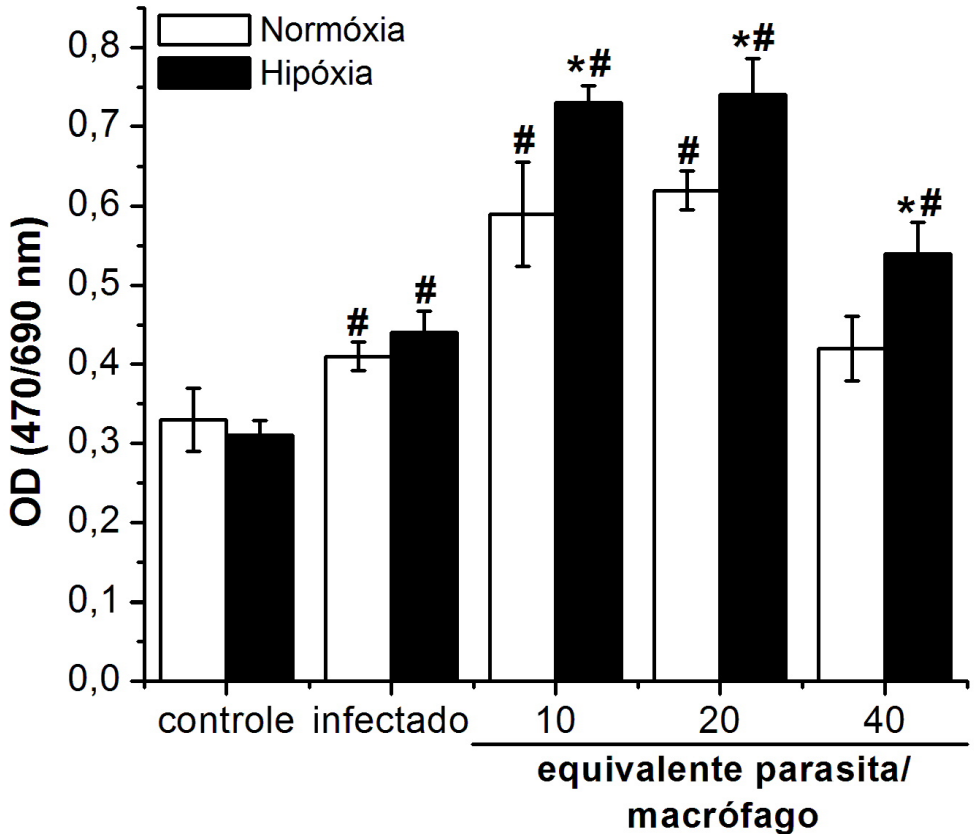


Figura 3. Capacidade de apresentação de antígeno dos macrófagos. Macrófagos peritoneais foram infectados com amastigotas de *L. amazonensis* (infectados) por 24 h em normóxia + 4 h em normóxia ou hipóxia ou pulsados com antígenos de *L. amazonensis* em diferentes concentrações, por 4 h em hipóxia ou normóxia. Macrófagos foram deixados sem infecção ou sem antígeno como controle. Após 4 h em normóxia ou hipóxia as culturas de macrófagos foram lavadas e as células de linfonodos de camundongos previamente imunizados com antígenos de *L. amazonensis* foram co-cultivadas com os macrófagos por 96 h, em normóxia A proliferação dos linfócitos foi quantificada pelo ensaio do MTT, conforme "Metodologia", em que a densidade ótica (OD) obtida é proporcional a quantidade de células. Os resultados representam média ± DP de um experimento realizado em quintuplicata. #P < 0.05 em relação a cultura de macrófagos+linfócitos controle em normóxia. *P<0,05 entre normóxia e hipóxia.

DISCUSSÃO

Os macrófagos são fagócitos profissionais multifuncionais que, além de secretarem uma variedade de fatores regulatórios, enzimas e citocinas e apresentarem atividades anti-tumoral e microbicida (ADEREM; UNDERHILL, 1999; GREENBERG, 2001; MCCORMICK; LI; CALERO, 2000), possuem também a capacidade de apresentação de antígeno (revisto por HUME, 2008). O ambiente hipóxico é capaz de alterar várias funções dos macrófagos como, por exemplo, a produção de citocinas e o processo fagocítico, como demonstrado pelos experimentos descritos no item Resultados Não Publicados e também por outros autores (LEEPER-WOODFORD; MILLS, 1992; MURATA et al., 2002; MURDOCH; LEWIS, 2005). Foi demonstrado que a expressão de CD80 (molécula co-estimulatória de superfície, envolvida na apresentação de antígenos) está reduzida em monócitos humanos cultivados em hipóxia (LAHAT et al., 2003), sugerindo, então, que a função "apresentadora de antígeno" (APC) de macrófagos está alterada durante processos hipóxicos. Na leishmaniose, a apresentação de antígeno aos linfócitos é importante, pois a destruição do parasita ou o controle da sua proliferação nos camundongos infectados é dependente de linfócitos T, que reconhecem os antígenos de *Leishmania* no contexto das moléculas do complexo principal de histocompatibilidade (MHC) presentes nas células apresentadoras de antígenos, como os macrófagos (PRINA et al., 1993). Assim, avaliamos se a hipóxia modifica a ativação dos linfócitos primados com antígenos de *L. amazonensis* e a capacidade APC dos macrófagos pulsados com estes antígenos. As células dos linfonodos de camundongos imunizados são estimuladas por antígenos de *L. amazonensis* e proliferam *in vitro* (Fig. 1). Entretanto, as células dos linfonodos não proliferaram em hipóxia na presença do antígeno (Fig. 1), indicando que a hipóxia inibe o reconhecimento do antígeno pelos linfócitos ou inibe a proliferação destas células. Para testar a hipótese de que a hipóxia inibe a proliferação dos linfócitos, usamos mitógenos, que induzem a proliferação dos linfócitos sem a

necessidade de reconhecimento de antígeno através de APCs. Como demonstrado na Figura 2, a proliferação dos linfócitos é induzida por ConA (mitógeno específico de células T) em normóxia, mas é reduzida em situação de hipóxia. Assim, a hipóxia parece inibir a capacidade proliferativa dos linfócitos, mesmo na presença de estimuladores. Se a hipóxia impede o correto reconhecimento do antígeno pelos linfócitos é uma questão a ser determinada.

Os experimentos de apresentação de antígeno *in vitro* foram realizados com macrófagos peritoneais como APCs. Macrófagos pulsados com antígenos de *L. amazonensis* em normóxia e hipóxia foram capazes de apresentar esses antígenos aos linfócitos primados, avaliado pela linfoproliferação após de 96 h de cultura (Fig. 3). A ativação dos linfócitos por macrófagos pulsados foi dependente da concentração dos parasitas, sendo máxima quando equivalente a 10 ou 20 parasitas/macrófago (Fig. 3). Quando um número maior de parasitas foi utilizado (40 parasitas/macrófago), os linfócitos não proliferaram (Fig. 3). Este resultado pode ser explicado pelo fato de que macrófagos expostos a altas concentrações do parasita teriam o catabolismo do antígeno prejudicado ou teriam as moléculas co-estimulatórias ou co-receptores modulados negativamente, o que resultaria em ausência de resposta pelos linfócitos T, como discutido por Caulada-Benedetti et al. (1998).

Para testar se a hipóxia altera o processamento e apresentação do antígeno pelos macrófagos, estas células foram pulsadas com antígeno durante quatro horas em hipóxia e depois cultivadas com células de linfonodos em ambiente normóxico. A proliferação dos linfócitos cultivados com macrófagos pulsados em hipóxia foi maior do que a proliferação dos linfócitos cultivados com macrófagos pulsados em normóxia (Fig. 3). Esse resultado indica que a hipóxia deve alterar a capacidade APC dos macrófagos. De fato, Acosta-Iborra et al. (2009) também demonstraram que a hipóxia aumenta a capacidade APC de macrófagos, pois estas células induzem um aumento na proliferação e ativação de linfócitos T em hipóxia, quando comparado a

normóxia. Os autores também demonstraram que macrófagos cultivados em hipóxia apresentaram um aumento na expressão de moléculas co-estimulatórias e moléculas envolvidas na apresentação de antígeno, como CD40, CD86 e MHC de classe I, assim como houve uma indução de TCR/CD3ε ativa pela hipóxia (ACOSTA-IBORRA et al., 2009). O aumento da expressão das moléculas co-estimulatórias, assim como a maior ativação dos macrófagos pela hipóxia pode explicar o aumento da capacidade de apresentação de antígeno dos macrófagos, como discutido por Acosta-Iborra et al. (2009). As células dos linfonodos cultivadas com macrófagos infectados com *L. amazonensis* proliferaram (Fig. 3), indicando que houve apresentação de antígeno e ativação dos linfócitos. Porém, essa ativação dos linfócitos não foi tão eficiente como no caso da ativação de linfócitos por macrófagos pulsados com antígenos, tanto em normóxia quanto em hipóxia (Fig. 3). A capacidade APC de macrófagos infectados com *Leishmania* é, de fato, alterada pela presença do parasita vivo, como previamente demonstrado (CHAKRABORTY et al., 2005; KAYE et al., 1994; LYTTON; MOZES; JAFFE, 1993; PRINA et al., 1993;). Macrófagos infectados com *Leishmania* apresentaram diminuição da expressão de moléculas co-estimulatória, como B7-1 (KAYE et al., 1994) e alteração na fluidez da membrana, com rompimento de "lipid rafts" da membrana plasmática, que são necessários para correta apresentação do antígeno (CHAKRABOTY et al., 2005). Além disso, a presença do parasita dentro de compartimentos da via endocítica poderia interferir no processamento e na apresentação do antígeno, como um mecanismo de evasão do sistema imune (PRINA et al., 1993), o que explicaria a baixa ativação dos linfócitos por macrófagos infectados.

Concluímos, com os resultados obtidos, que a hipóxia reduz a capacidade proliferativa dos linfócitos T e altera o processamento e apresentação de antígeno de *L. amazonensis* pelos macrófagos. Como a apresentação de antígeno aos linfócitos é importante na leishmaniose, colaborando com a destruição do parasita ou o controle da sua proliferação nos camundongos

infectados, sugere-se que a hipóxia presente nas lesões possa interferir no processamento e apresentação do antígeno pelos macrófagos e na proliferação e ativação dos linfócitos T, podendo contribuir para a dinâmica da doença.

5. CONCLUSÕES

- O cultivo de macrófagos em ambiente hipóxico causa alterações nestas células, como produção de ROS e de citocinas (TNF-α, IL-6 e IL-12) e alteração no processo fagocítico, indicando que a hipóxia estimula estes macrófagos (ver Manuscrito).

- Dentre os parâmetros avaliados para se estabelecer o mecanismo de redução da infecção de macrófagos pela *L. amazonensis* em hipóxia, óxido nítrico (NO), citocinas (TNF-α, IL-6, IL-12, IL-10), comprometimento metabólico e os processos de fagocitose ou exocitose do parasita provavelmente não são responsáveis por este efeito leishmanicida da hipóxia. ROS são importantes para o efeito leishmanicida da hipóxia, embora não se saiba o exato mecanismo da sua atuação (ver Manuscrito).

- Macrófagos infectados em hipóxia não induzem a morte tipo apoptose nos amastigotas intracelulares (ver Manuscrito).

- A expressão das isoformas HIF-1α e HIF-2α é estabilizada nos macrófagos infectados pelo parasita *L. amazonensis* em situação normóxica. A indução deste fator transcricional e de seus genes alvos provavelmente constitui parte do mecanismo adaptativo do macrófago resultante da infecção pelo parasita (ver Manuscrito e o Artigo em anexo).

- A hipóxia altera a capacidade proliferativa dos linfócitos T, assim como altera o processamento e apresentação de antígeno de *L. amazonensis* pelos macrófagos. Estes dados obtidos *in vitro* podem ser extrapolados para a situação *in vivo*, sugerindo que macrófagos que processam o antígeno de *L. amazonensis* em situação hipóxica pode ser alterado, influenciando o curso da lesão através da apresentação de antígeno aos linfócitos T (ver Proliferação de linfócitos e apresentação de antígenos por macrófagos em hipóxia).

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ANEXO

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

Adriana Degrossoli, Maira Cegatti Bosetto, Camila Bárbara Cantalupo Lima, Selma Giorgio *

Departamento de Parasitologia, Instituto de Biologia, Universidade Estadual de Campinas, Caixa Postal 6109, 13083-970 Campinas, São Paulo, Brazil

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Abstract

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

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

1. Introduction

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

2. Materials and methods

2.1. Parasites

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

* Corresponding author. Tel.: +55 19 35216287; fax: +55 19 35216282.
E-mail address: sgiorgio@unicamp.br (S. Giorgio).

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

2.2. Cell culture

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

2.3. Normoxic and hypoxic conditions

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

2.4. Macrophage infection

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

2.5. Cadmium chloride ($CdCl_2$) assay

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

2.6. HIF-1α immunofluorescence

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

2.7. Detection of cellular hypoxia

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

2.8. Results analyses

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

3. Results

3.1. HIF-1α immunofluorescence analyses

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

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

3.2. Immunofluorescence analyses with pimonidazole as a hypoxic marker

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

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

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

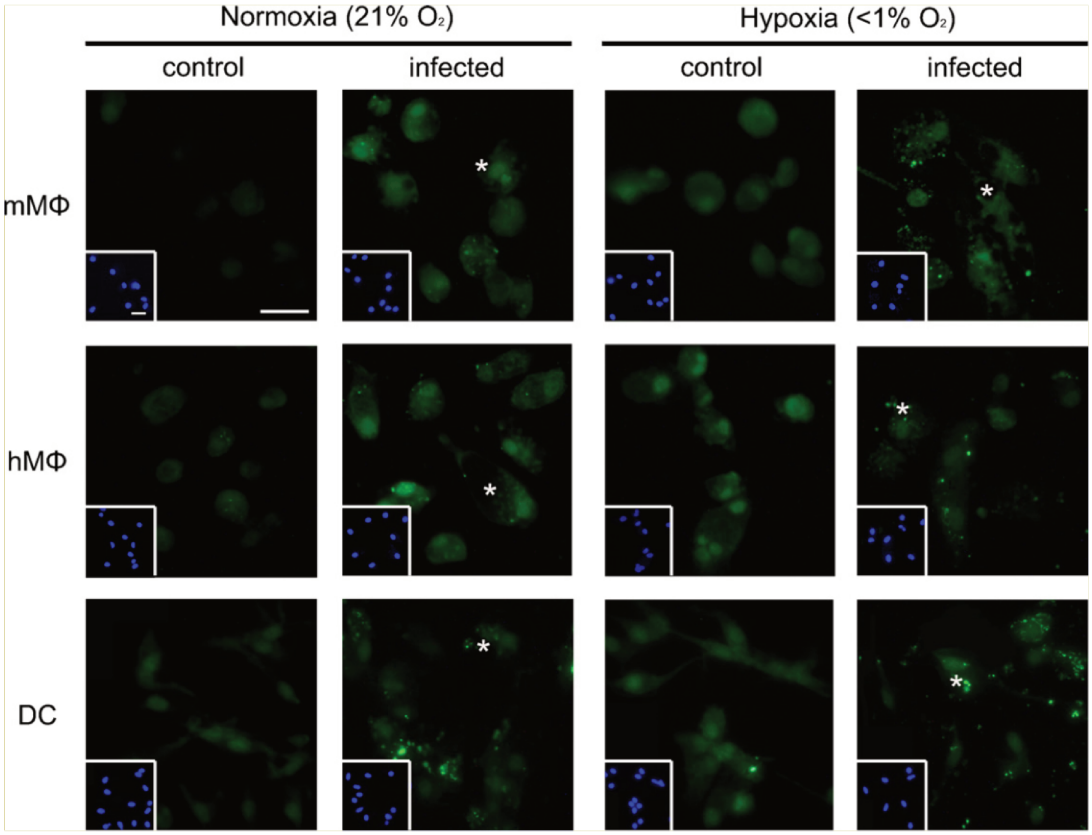

Fig. 1. Expression of HIF-1α by mouse macrophages (mMΦ) and human macrophages (hMΦ) and human dendritic cells (DC). The cells were infected with *L. amazonensis* amastigotes or left uninfected (control) and exposed to normoxia or hypoxia for 24 h, as described in Section 2. The cells were fixed and stained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

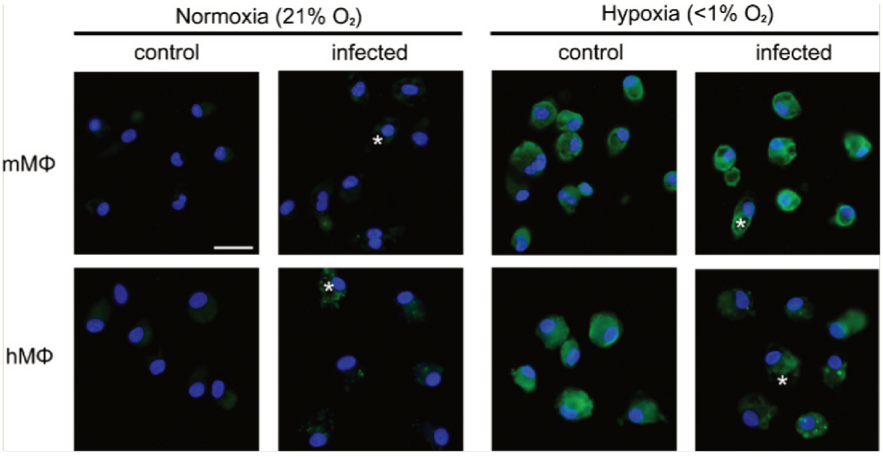

Fig. 2. Pimonidazole staining of mouse macrophages (mMΦ) and human macrophages (hMΦ). Cells were infected with *L. amazonensis* amastigotes or left uninfected (control), treated with pimonidazole (200 μM) and exposed to normoxia or hypoxia for 24 h as described in Section 2. The cells were fixed and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

4. Discussion

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

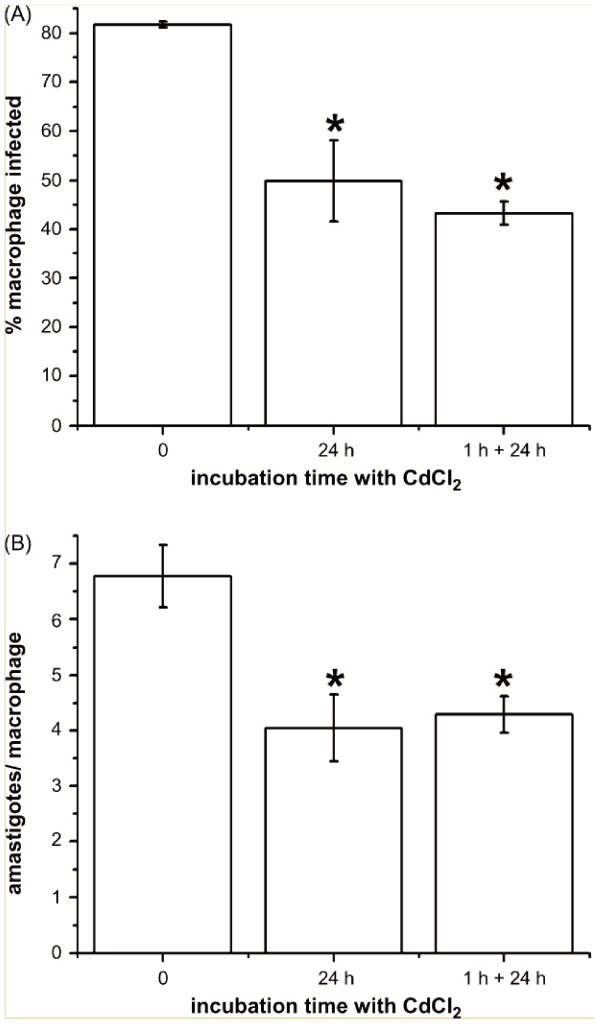

Fig. 3. Effect of $CdCl_2$ on *L. amazonensis*-infected MΦ. Mouse MΦ were infected with *L. amazonensis* amastigotes for 1 h and then washed to remove extracellular parasites. Twenty-five micromolars of $CdCl_2$ were added to the cell cultures, which were incubated in normoxia at 37 °C for 24 h (24 h).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

our group showed that HIF-1α is expressed in mouse cutaneous lesions during infection with *L. amazonensis*, an exclusive parasite of mononuclear phagocytes [5]. In this report, the expression of HIF-1α and a chemical marker of hypoxia (pimonidazole) in primary cultures of MΦ and DC infected with *L. amazonensis*, were analyzed. These in vitro cell systems are very useful for studying the cell biology of the host–parasite interactions [38,39]. In the experiments reported here, mMΦ, hMΦ and hDC were easily infected with *L. amazonensis* amastigote or promastigote forms and supported the infection without rapid cell degeneration. Large parasitophorous vacuoles, typically produced by this *Leishmania* species in MΦ and DC, facilitated microscopic analyses by visualization and counting [39,40]. Before elaborating on HIF-1α expression in *L. amazonensis*-infected mononuclear phagocytes, it should be noted that, to the best of our knowledge, these are the first experiments concerning the examination of HIF-1α expression in DC. It was of some interest to observe that HIF-1α was detected in the nucleus of noninfected DC under normoxic conditions, whereas HIF-1α was not detected in noninfected mMΦ and hMΦ when cultured in normoxia. This parallels the findings that HIF-1α is present at detectable levels under normoxia in some skeletal cells and during PMA-mediated differentiation in monocytic cell lines (THP1 and U937) [41–43]. In addition, cytokines, such as TNF-α, interferon-γ, IL1β and IL4 increase HIF-1α mRNA and/or protein levels during normoxia [44]. Ascertaining whether HIF-1α expression in DC under normoxic conditions is associated with the presence of IL4 and GM-CSF in the culture medium during the induction of DC differentiation should be the object of further investigation.

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

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

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DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha tese de Doutorado intitulada "Estudos de alterações funcionais de macrófagos submetidos a hipóxia no modelo *in vitro* da leishmaniose":

( ) não se enquadra no § 3° do Artigo 1° da Informação CCPG 01/08, referente a bioética e biossegurança.

( ) está inserido no **Projeto CIBio/IB/UNICAMP** (Protocolo n°_____), intitulado _____;

(X) tem autorização da **Comissão de Ética em Experimentação Animal/IB/UNICAMP** (Protocolo n° 1741-1);

( ) tem autorização do **Comitê de Ética para Pesquisa com Seres Humanos/FCM/UNICAMP** (Protocolo n°_____);

( ) tem autorização de comissão de bioética ou biossegurança externa à UNICAMP.
Especificar: _____

Adriana Degrossoli
Aluno: Adriana Degrossoli

Selma Giorgio
Orientador: Selma Giorgio

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(X) Deferido ( ) Indeferido

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