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# Regulation of the expression of nitric oxide synthase by *Leishmania mexicana* amastigotes in murine dendritic cells

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ABSTRACT

In mammalian hosts, *Leishmania* parasites are obligatory intracellular organisms that invade macrophages (M$\phi$) and dendritic cells (DC). In M$\phi$, the production of nitric oxide (NO) catalyzed by the inducible nitric oxide synthase (iNOS) has been implicated as a major defense against *Leishmania* infection. The modulation of this microbicidal mechanism by different species of *Leishmania* has been well studied in M$\phi$. Although DC are permissive for infection with *Leishmania* both *in vivo* and *in vitro*, the effect of this parasite in the expression of iNOS and NO production in these cells has not been established. To address this issue, we analyzed the regulation of iNOS by *Leishmania mexicana* amastigotes in murine bone marrow-derived dendritic cells (BMDC) stimulated with LPS and IFN-$\gamma$. We show that the infection of BMDC with amastigotes down regulated NO production and diminished iNOS protein levels in cells stimulated with LPS alone or in combination with IFN-$\gamma$. The reduction in iNOS protein levels and NO production did not correlate with a decrease in iNOS mRNA expression, suggesting that the parasite affects post-transcriptional events of NO synthesis. Although amastigotes were able to reduce NO production in BMDC, the interference with this cytotoxic mechanism was not sufficient to permit the survival of *L. mexicana*. At 48 h post-infection, BMDC stimulated with LPS + IFN-$\gamma$ were able to eliminate the parasites. These results are the first to identify the regulation of iNOS by *L. mexicana* amastigotes in DC.



## 1. Introduction

*Leishmania* are dimorphic protozoan parasites that live and replicate in the gut of sandflies as flagellated forms (promastigotes) or as aflagellated forms (amastigotes) in mammalian hosts, where they become obligate intracellular parasites that preferentially infect phagocytic cells, primarily macrophages (M$\phi$) and dendritic cells (DC)[1]. DC are a heterogeneous group of antigen presenting cells that can be divided into subtypes based on surface phenotype and function (Heath et al., 2004; Shortman and Liu, 2002). The properties of DC vary with the different stages of their life span. In nonlymphoid tissues they are present as immature cells ready to detect, phagocytose, and process antigens to transport them to lymph nodes. There, DC present antigens to naive T cells to initiate and coordinate an adaptive immune response.

Professional phagocytic cells produce a number of microbicidal compounds, aiding the control of microbial intracellular proliferation. Amongst these, nitric oxide (NO) has been described as a potent cytotoxic and cytostatic agent in cell-mediated immune responses capable of limiting the growth of several intracellular microorganisms. Production of NO by eukaryotic cells is catalyzed by at least three isoforms of nitric oxide synthase (NOS), which convert L-arginine and molecular oxygen to L-citrulline and NO. The type 2 isoform (NOS2), also known as inducible nitric oxide synthase (iNOS), is characterized by a high-output production of NO and an extensive regulation on the transcriptional and post-transcriptional levels by various stimulatory and inhibitory cytokines. In M$\phi$, the prototypic cell type for NOS2-dependent synthesis of NO, NOS2 mRNA and protein are undetectable unless the cells are activated by interferon $\gamma$ (IFN-$\gamma$) and/or lipopolysaccharide (LPS) (MacMicking et al., 1997a,b). Expression of NOS2 by M$\phi$ has been closely associated with the killing of intracellular microbes (e.g., cryptococci, *Toxoplasma*, mycobacteria, and *Leishmania*) *in vitro* and with the resolution or an ameliorated course of the respective infections *in vivo* (Bogdan, 1997; MacMicking et al., 1997a,b; Scharton-Kersten et al., 1997). In the case of *Leishmania major*, the hallmark of a protective immune response against this protozoan is the induction and expansion of $CD4^+$ type 1 T helper lymphocytes (Th1), which activate M$\phi$ via the production of IFN-$\gamma$ for the killing of intracellular amastigotes (Reiner and Locksley, 1995). In mice infected with *L. major* promastigotes, the

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[1] *Abbreviations used:* DC, dendritic cells; NO, nitric oxide; iNOS, inducible nitric oxide synthase; BMDC, bone marrow-derived dendritic cells.

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ultimate healing of the cutaneous lesions and the control of life-long persistent parasites during the chronic phase of infection were dependent on the activity of NOS2 in the tissue (Stenger et al., 1996). In spite of the potent leishmanicidal mechanisms of M$\phi$, *Leishmania* can infect and multiply in these cells. Several mechanisms may explain the resistance of *Leishmania* to the M$\phi$ defense response (Balestieri et al., 2002). Among these, the characteristic inhibition of macrophage IL-12 production following *L. major* infection may be an important parasite survival strategy by delaying IFN-$\gamma$ production and down regulating activation of macrophages for NO production (Carrera et al., 1996). In addition, the production of TGF-$\beta$ by peritoneal M$\phi$ infected with *L. amazonensis* or *L. braziliensis* inhibits NO synthesis (Balestieri et al., 2002; Barral et al., 1993; Barral-Netto et al., 1992).

Although DC can phagocytose *Leishmania*, preferentially amastigotes (Bennett et al., 2001; Prina et al., 2004), it is unclear whether *Leishmania* can survive within these cells (Blank et al., 1993; Marovich et al., 2000; von Stebut et al., 1998, 2000). In addition to the essential role of DC in stimulating the immune system, they probably act as effector cells in the first line of defense against pathogen invasion. DC act as "sentinels" in the immunological response to various infecting agents such as bacteria (Svensson et al., 1997), viruses (Bhardwaj et al., 1994), and parasites (Aline et al., 2002). It has been shown that NO produced by DC is an important factor restricting the intracellular survival of *Salmonella enterica* sv. Typhimurium (Eriksson et al., 2003). In order to explore the effect of *Leishmania* parasites in NO synthesis by DC, we analyzed nitrites production, iNOS mRNA, and protein levels in bone marrow-derived dendritic cells (BMDC) infected with *Leishmania mexicana* amastigotes and stimulated with LPS and IFN-$\gamma$. We found that amastigotes decreased the protein levels of iNOS and production of NO in BMDC stimulated with LPS and LPS + IFN-$\gamma$. This correlates with what has been found about the functional impairment of DC activation by *L. amazonensis* (Xin et al., 2008) and *L. mexicana* (Bennett et al., 2001) amastigotes. Nevertheless, BMDC were able to eliminate intracellular amastigotes 48 h post-infection, which suggests the participation of other mechanisms in the clearance of parasites by DC.

## 2. Materials and methods

### 2.1. Animals

Female and male C57BL/6 and BALB/c mice were purchased from Harlan Laboratories (Indianapolis, IN, USA) and bred in the animal facility at the Departamento de Medicina Experimental, Facultad de Medicina, UNAM, following the national guidelines for animal care. Mice were used at 6–10 week of age.

### 2.2. Culture of Leishmania mexicana amastigotes

*L. mexicana* M-379 strain (MNYC/BZ/62/M379; ATCC 50156) was used throughout the experiments and was kindly provided by Dr. Eric Dumonteil and Dra. Ma. del Rosario García (Centro de Investigaciones Regionales, CIR, Dr. Hideyo Noguchi, UADY, Mérida, Yuc., México). Amastigotes were isolated from footpad lesions of BALB/c mice infected 6–8 week earlier, as previously described (Hart et al., 1981; Mosser and Edelson, 1985; Yang et al., 2007), with some modifications. Briefly, the plunger of a 10-ml syringe was used to pass the excised foot through a 100 μm nylon cell strainer (BD Falcon, Bedford, MA, USA) in the presence of pyrogen-free and sterile Schneider's *Drosophila* insect cell culture medium (Sigma, St. Louis, MO, USA) supplemented with 20% (v/v) heat-inactivated certified endotoxin tested-fetal bovine serum (FBS), 25 μg/ml gentamicin sulphate, and 2 mM L-glutamine (Gibco Invitrogen Corporation, Carlsbad, CA, USA) with a pH of 5.4 adjusted with 1.0 M HCl. Release of amastigotes from infected cells was achieved by passing the mixture through progressively smaller 21-, 23-, and 25-gauge needles. Footpad-derived amastigotes were obtained by centrifugation at 2000*g* for 10 min and cultured at 32 °C, as previously described (Bates et al., 1992). Amastigotes cultures were started by seeding $5 \times 10^5$ cells/ml in 25 $cm^2$ tissue culture flasks in a final volume of 10 ml and subcultured every 5–6 days.

### 2.3. Bone marrow-derived dendritic cells (BMDC)

Mice were sacrificed by cervical dislocation following humane handling, as established by the ethical committee of the Facultad de Medicina, UNAM.

BMDC were prepared as described by Lutz et al. (1999), with some modifications. Briefly, bone marrow was flushed from the femurs and tibiae of eight-week old C57BL/6 mice and plated in 100-mm bacteriological Petri dishes at a concentration of $2 \times 10^6$ leukocytes in 10 ml of medium. Cell culture medium was pyrogen-free and sterile RPMI-1640 medium containing 10% heat-inactivated certified endotoxin tested-fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-Mercaptoethanol (Gibco Invitrogen Corporation, Carlsbad, CA, USA), 25 mM HEPES buffer, and 24 mM $NaHCO_3$ (Sigma–Aldrich Co., St. Louis, MO, USA). Complete medium was supplemented with 10% GM–CSF-containing supernatant from culture of X-63 cell line transfected with the murine GM–CSF gene, found to be equivalent to 200 U/ml of GM–CSF (Zal et al., 1994). The X-63 cell line was kindly donated by Dr. Brigitta Stockinger (Medical Research Council, Mill Hill, London, UK). On day 3, 10 ml of GM–CSF-containing medium were added to each dish and on day 6, 10 ml of the culture medium were replaced with fresh GM–CSF-containing medium. On day 8, nonadherent cells were harvested, centrifuged, resuspended in RPMI complete medium, and used for the experiments. By flow cytometry, cells were generally 85% $CD11c^+$ pure and had a $CD11c^+CD11b^+$ phenotype, with only moderate expression of MHC II and CD86.

### 2.4. FACS analysis

Cells were harvested from plates and resuspended at $1 \times 10^6$/100 μl of PBS supplemented with 2% heat-inactivated horse serum and 5 mM EDTA. Cells were stained for 30 min at 4 °C with 1:200 dilution of the following antibodies: APC anti-mouse CD11c, PE anti-mouse I-A/I-E (BD Pharmingen, San Jose, CA, USA), FITC anti-mouse CD11b, and FITC anti-mouse CD86 (eBioscience, San Diego, CA, USA). Cells were washed 3× with PBS and fixed in 2% paraformaldehyde for 30 min at 4 °C. Cells were washed and analyzed with a Becton–Dickinson FACSCalibur apparatus (Becton Dickinson, San Jose, CA, USA) using the CellQuest software.

### 2.5. Parasite infection and cell stimulation

BMDC were plated at a concentration of $1 \times 10^6$ cells/ml of RPMI complete medium in 24-well culture plates (Corning Life Sciences, NY, USA). After resting for 2 h at 37 °C in 5% $CO_2$, cells were infected with *L. mexicana* amastigotes at 10:1 parasite/cell ratio for 4 h at 32 °C. Afterwards, cells were stimulated with 10 ng/ml of lipopolysaccharide (LPS) of *Escherichia coli* (*E. coli* serotype 0111:B4 Sigma, St. Louis MO, USA) and/or 100 U/ml of murine recombinant IFN-$\gamma$ (Preprotech, Rock Hill, NJ, USA) and were incubated at 37 °C for 6 h for RT–PCR, 24 h for Western blot and 24 or 48 h for NO measurement.