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Leishmania (Viannia) braziliensis amastigotes induces the expression of TNFα and IL-10 by human peripheral blood mononuclear cells *in vitro* in a TLR4-dependent manner

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ABSTRACT

While the role of Toll-like receptors (TLRs) has been investigated in murine models of tegumentary leishmaniasis caused by *Leishmania (Viannia) braziliensis*, the interaction between TLRs and *Leishmania* sp. has not been investigated in human cells. The aim of this study was to evaluate the involvement of TLR4 in cytokine production of human peripheral blood mononuclear cells (PBMCs) induced by *L. braziliensis*, and whether the parasite alters the expression of TLR4 on monocytes/macrophages. Amastigote forms were obtained from mice lesions and PBMCs were isolated from healthy donors. PBMCs were cultured in absence or presence of IFNγ, TLR4 neutralizing antibodies, natural antagonist of TLR4 (*Bartonella* LPS), TLR4 agonist (*E. coli* LPS), and amastigote forms. The concentrations of tumor necrosis factor (TNFα) and interleukin 10 (IL-10) were assayed by ELISA and TLR4 expression by flow cytometry. Amastigotes forms of *L. braziliensis* induced TNFα and IL-10 production only in IFNγ-primed PBMCs. The TNFα and IL-10 production was inhibited by TLR4 neutralization, both with anti-TLR4 antibodies and *Bartonella* LPS. Interestingly, addition of *E. coli* LPS further increased TNFα but not IL-10 production induced by *L. braziliensis* amastigotes. Amastigotes of *L. braziliensis* strongly reduced membrane TLR4 expression on monocytes/macrophages, apparently by internalization after the infection. The present study reveals that TLR4 drives the production of TNFα and IL-10 induced by *L. braziliensis* amastigotes and that the parasites decrease TLR4 expression on monocyte surface.

1. Introduction

American Tegumentary Leishmaniais (ATL) is a parasitic disease caused by protozoa *Leishmania* sp. infection of phagocytic mononuclear cells, characterized by lesions in skin and oral or nasopharyngeal mucosa [1]. In the Americas alone 66,941 cases of cutaneous leishmaniasis are reported each year with an estimated annual incidence of 187,200 to 307,800 cases [2]. ATL manifests as several clinical forms, depending on the parasite species and the host immune response. Among seven species of *Viannia* subgenus in Brazil, *L.* (*Viannia*) *braziliensis* and *L.* (*V.*) *guyanensis* cause ATL, leading to distinct clinical forms such as localized cutaneous leishmaniasis (LCL), disseminated cutaneous leishmaniasis (DCL) and mucosal leishmaniasis (ML). Of these species, infection by *L. braziliensis* is most prevalent [1,3]. Both species can be infected by RNA virus (LRV-1) and the presence of the virus is associated with development of ML [4,5].

The interactions between microorganisms and cells from the innate immune system involve toll-like receptors (TLRs) that recognize pathogen-associated molecular patterns (PAMPs), including glycolipids, peptidoglycans, lipopeptides and nucleic acids. The signaling pathways activated by TLRs are central to adaptive immune responses influencing disease outcome [6]. In Leishmaniasis, monocytes and macrophages release several cytokines after recognition of *Leishmania* parasites such as tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ) and interleukin (IL)-10. Both

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TNFα and IFNγ play important roles in inflammation and parasite control whereas IL-10 acts as anti-inflammatory and immunosuppressor cytokine [7]. *Leishmania* membrane contains glycoinositol phospholipids (GIPLs), lipophosphoglycans (LPG) and glycoprotein gp63 as main molecules recognized by the innate immune system [8]. It has been previously demonstrated that TLR2 is engaged by LPG purified from *L. major* [9], *L. infantum* and *L. braziliensis* promastigotes [10], inducing nitric oxide (NO) and cytokines (TNFα, IL-1β, L-6) in murine macrophages. *L. braziliensis* LPG is a strong agonist of TLR2 [10], and similarly *L. major* LPG triggers TLR2 in human NK cells to enhance IFNγ and TNFα production [11] and induces reactive oxygen species (ROS) production, IFNγ, and interleukin (IL)-12 in human peripheral blood mononuclear cells (PBMCs) [12]. In addition, parasites infected with the LRV1 RNA virus can activate TLR3 leading to increased inflammation [5]; however, despite of *L. guyanensis* can be infected by LRV1, mouse protection against this parasite species is mediated by TLR9 activation [13].

Best characterized as a receptor for lipopolysacchsaride (LPS) of Gram negative bacteria [14], TLR4 also recognize other PAMPs, including glycolipids of protozoan [15] such as *L. infantum* and *L. braziliensis* GIPLs [16] and *L. amazonensis* LPG [17]. TLR4 activation with GIPLs or LPG in murine macrophages leads to NO and TNFα production [16,17]. Furthermore, TLR4 plays an important role in murine *L. major* infection [18]. Compared to wild type mice, TLR4 knockout macrophages are more permissive to parasites due to reduced NO production. This is intensified by IL-4, which increases arginase activity to favor parasite growth [19]. In contrast, TLR4 is used by *L. mexicana* promastigotes to induce arginase but not NO in murine macrophages [20]. TLR4 is crucial to TNFα induction by *L.* (*V.*) *panamensis* in murine cells, however endosomal TLRs also can be involved [21]. Therapeutic effects of TLR4 and TLR9 activation are associated with induction of efficient cellular immune responses in murine tegumentary as well as in visceral leishmaniasis [22–24]. In mice, agonists of TLR4 and TLR9 improve resistance to parasites causing Old and New World cutaneous leishmaniasis in immunoprophylactic or immunotherapeutic manner [22,25].

Important research is beginning to elucidate the role of TLR signaling in infections by *L. braziliensis*. MyD88-defficient mice are more susceptible to *L. braziliensis* infection than wild type mice, suggesting that TLR signaling is important for parasite control. Absence of MyD88 leads to less activation of dendritic cells and decreased production of IL-12, resulting in increased lesion size and more prolonged disease in comparison to wild type mice [26]. In this study, absence of TLR2 enhanced the resistance to infection, suggesting that TLRs other than TLR2 could be involved in immune response against *L. braziliensis*. Recently, involvement of TLR9 was demonstrated in activation of dendritic cells by *L. braziliensis* promastigotes. TLR9-deficient mice exhibited larger lesions and higher parasite burden than wild type mice, especially during the first weeks of infection [27]. Interestingly, polymorphisms in the Toll-interacting protein (TOLLIP) gene are associated with greater susceptibility to cutaneous leishmaniasis in patients from north of Brazil [28].

Experiments aimed to explore the role of TLR4 using either TLR4 deficient mice or primary human cells infected with *L. braziliensis* are lacking at this moment. Expression of human TLR2, TLR4 and TLR9 has been demonstrated in lesions of Leishmaniasis patients [29,30] and TLR9 was also detected in *L. braziliensis* antigen-activated monocytes [31] of ATL patients infected with *L. braziliensis*. Based on the limited knowledge about *Leishmania* and TLR4 interaction in human cells, the aim of the present study was to investigate the role of TLR4 in the induction of TNFα and IL-10, in human PBMCs infected with *L. braziliensis* amastigotes. In addition, we evaluated whether amastigotes were able to modulate the expression of TLR4 on human monocytes/macrophages.

2. Materials and methods

2.1. Obtaining of human peripheral blood samples and animals

The study was approved by Ethical Committee of Federal University of Goiás (protocol N.165/08), and included male or female healthy donors from Blood Banks (Hospital das Clínicas/Federal University of Goiás and Instituto Goiano de Oncologia e Hematologia), who consented to participate in the research. Donors were between 18 and 60 years old (n = 26). Venous blood was collected in EDTA-vacutainer tubes (BD Vacutainer™, MG, Brazil). IFNγ knockout C57BL/6 mice (n = 15) were maintained in facility of the Institute of Tropical Pathology and Public Health, under specific pathogen free conditions, with 12 h-light cycles.

2.2. Peripheral blood mononuclear cells (PBMCs) and amastigote isolation

PBMCs were obtained from blood samples and were overlaid on density gradient (Ficoll, GE Healthcare, UK), centrifuged at room temperature (1500 g, 20 min). After two washings of PBMCs (0.01 M EDTA in phosphate buffer saline (PBS), pH 7.2; 700 g, 10 min; and 200 g, 10 min), cells were suspended in RPMI 1640 medium (Sigma-Aldrich, MD, USA) supplemented with 10% fetal calf serum (FCS, Gibco, Life Technologies, SP, Brazil), 2 mM L-glutamine, 11 mM sodium bicarbonate, 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich). Cell viability was validated with 0.01% trypan blue in PBS and PBMCs were distributed (1.5×10^6 cells/400 μL) into 24-well plates (Corning Costar, NY, EUA).

MHOM/BR/2003/IMG *L. braziliensis* (IMG3) parasites were obtained from cutaneous lesion of LCL patient (*Leishbank* IPTSP/UFG), as previously described [32], and confirmed as negative for LRV-1 (Supplementary Fig. S1), were cultured in Gracés medium (Grace's Insect Medium, Sigma-Aldrich) supplemented with inactivated 20% FCS (Gibco), 2 mM L-glutamine (Sigma-Aldrich), 100 U/mL penicillin (Sigma-Aldrich) and 100 μg/mL streptomycin (Sigma-Aldrich). Parasites from stationary phase of growth (6º day) were washed three times (PBS, 1500 g, 15 min, 10 °C) and diluted into 0.1% formaldehyde (Synth, SP, Brazil) in PBS for quantification in hemocytometer. Parasites were inoculated (5×10^6) into mice footpads to obtain amastigotes according to previously described [33,34]. Briefly, lesions (>1 mm; ~3–4 weeks post infection) were collected from euthanized mice and macerated in PBS. Tissue debris were washed out with PBS (114 g, 5 min, 10 °C) and cell suspension (1500 g, 15 min, 10 °C) was subjected to red blood cell lyses (90% NH_4Cl 0.16 M; 10% Tris 0.17 M) followed by another washing with PBS (1500 g, 15 min, 10 °C). Amastigotes were separated through 100% and 44% Percoll gradient (GE Helthcare, USA), following centrifugation (2500 g, 30 min, 10 °C). After one washing with PBS (2500 g, 20 min, 10 °C), amastigotes were suspended in PBS and quantified using a hemocytometer.

2.3. PBMCs cultures and treatments

PBMCs (1.5×10^6 cells/400 μL) were cultured in absence or presence of human recombinant IFNγ (100 U/mL [5 ng/mL], Preprotech, Ribeirão Preto, SP, Brazil) for 24 h, at 37 °C and 5% CO_2. IFNγ-treated PBMCs were incubated in absence or presence of neutralizing antibodies to TLR4 (100 μg/mL; HTA125, Biolegend, San Diego, CA, USA) or *Bartonella quintana* LPS (BartLPS, 5 μg/mL), a natural antagonist of TLR4 receptor [35], 1 h before addition of amastigotes (3×10^5 parasites/well, at a parasite to PBMC ratio of 1:5 or ~2:4, considering 5 to 10% of monocytes in PBMCs). IFNγ-treated PBMCs cultured in absence or presence of amastig-