IL-1β Production by Intermediate Monocytes Is Associated with Immunopathology in Cutaneous Leishmaniasis

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Cutaneous leishmaniasis due to *Leishmania braziliensis* infection is an inflammatory disease in which skin ulcer development is associated with mononuclear cell infiltrate and high levels of inflammatory cytokine production. Recently, NLRP3 inflammasome activation and IL-1β production have been associated with increased pathology in murine cutaneous leishmaniasis. We hypothesized that cutaneous leishmaniasis patients have increased expression of NLRP3, leading to high levels of IL-1β production. In this article we show high production of IL-1β in biopsy samples and *Leishmania* antigen-stimulated peripheral blood mononuclear cells from patients infected with *L. braziliensis* and reduced IL-1β levels after cure. IL-1β production positively correlated with the area of necrosis in lesions and duration of the lesions. The main source of IL-1β was intermediate monocytes ($CD14^{++}CD16^{+}$). Furthermore, our murine experiments show that IL-1β production in response to *L. braziliensis* was dependent on NLRP3, caspase-1, and caspase-recruiting domain (ASC). Additionally, we observed an increased expression of the *NLRP3* gene in macrophages and the NLRP3 protein in intermediate monocytes from cutaneous leishmaniasis patients. These results identify an important role for human intermediate monocytes in the production of IL-1β, which contributes to the immunopathology observed in cutaneous leishmaniasis patients.

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INTRODUCTION

Cutaneous leishmaniasis (CL) by *Leishmania braziliensis* is characterized by the presence of one or more well-limited ulcers with raised borders and only a few parasites (Llanos Cuentas et al., 1984; Saldanha et al., 2017). In the initial phase of the disease, known as early cutaneous leishmaniasis (ECL), most patients develop regional lymphadenopathy and a nonulcerated lesion, and as disease progresses the inflammatory infiltrate is composed mainly of T and B lymphocytes, plasma cells, and mononuclear phagocytes (Bittencourt and Barral, 1991; Dantas et al., 2014; Saldanha et al., 2017). The inflammatory response, crucial to control parasite replication, also drives tissue damage, leading to skin ulcer development (Antonelli et al., 2005; Carvalho et al., 2007, 2012; Ribeiro-de-Jesus et al., 1998). In this manner, $CD8^{+}$ T cells have been described as one of the main cells driving immunopathology in CL lesion sites, whereas $CD4^{+}$ T cells are associated with protection (Cardoso et al., 2015; da Silva Santos et al., 2013; Novais et al., 2017).

Less attention, however, has been given to the contribution of mononuclear phagocytes to protection or immunopathology during *L. braziliensis* infection. Circulating monocytes constitute a heterogeneous population of cells, and based on the surface expression of CD14 and CD16, these cells can be subdivided into classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and nonclassical ($CD14^{+}CD16^{++}$) subsets (Almeida et al., 2017; Zawada et al., 2011; Ziegler-Heitbrock, 2010, 2015). Monocyte subsets differ not only phenotypically but also functionally as intermediate monocytes that have been associated with immunopathology in rheumatoid arthritis, sepsis, and CL (Fingerle et al., 1993; Kawanaka et al., 2002; Passos et al., 2015). We have found that early after infection and before ulceration is established, the frequency of circulating CD16-expressing monocytes (intermediate and nonclassical) are increased in the peripheral blood of *L. braziliensis*-infected

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Abbreviations: CCL, cured of cutaneoul leishmaniasis; CL, cutaneous leishmaniasis; ECL, early cutaneous leishmaniasis; FBS, fetal bovine serum; HS, healthy subject; PBMC, peripheral blood mononuclear cell

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individuals (Passos et al., 2015). Our data also show that intermediate monocytes were the main source of TNF, an inflammatory cytokine for which levels are increased during CL and that is highly associated with tissue damage and lesion development (Passos et al., 2015). These data argue in favor of a deleterious role for intermediate monocytes in CL.

IL-1β is a key cytokine of inflammation produced by mononuclear phagocytes participating in the pathogenesis of various diseases such as autoimmune, autoinflammatory, metabolic, and neurodegenerative diseases contributing to the severity of the pathological process (Dinarello, 2009, 2012; Ferrari et al., 2006; Halle et al., 2008; Roerink et al., 2017). IL-1β is produced as an inactive cytoplasmic precursor and is cleaved by caspase-1 in a process normally dependent or independent of inflammasome activation to become biologically active (Martinon et al., 2002; Netea et al., 2009). The formation of inflammasome complexes is an important proinflammatory pathway of the innate immunity (Clay et al., 2014). The NLRP3 inflammasome has the best studied list of agonists, such as low intracellular K^+ concentration; extracellular adenosine triphosphate; and viral, bacterial, and parasitic pathogens (Clay et al., 2014; Kang et al., 2017; Katsnelson et al., 2015; Kuriakose and Kanneganti, 2017; Pétrilli et al., 2007; Piccini et al., 2008). We and others have recently shown the importance of the NLRP3 inflammasome in the immunopathogenesis of leishmaniasis and shown that NLRP3 is directly involved in processing and releasing of IL-1β during infection with *Leishmania* species in mouse models (Almeida et al., 2017; Charmoy et al., 2016; Gurung and Kanneganti, 2016; Gurung et al., 2015; Novais et al., 2017). Rather than be involved in parasite killing, reports have shown a function for IL-1β in contributing to the development of the inflammatory process in leishmaniasis, exacerbating disease (Charmoy et al., 2016; Novais et al., 2017).

In this article we documented that intermediate monocytes from CL patients secrete IL-1β and express NLRP3 and that IL-1β production increases with CL progression. Furthermore, the amount of IL-1β positively correlates with areas of necrosis and time of lesion in CL patients. Moreover, our results in mice showed that the NLRP3 inflammasome was the pathway involved in IL-1β production.

RESULTS

IL-1β is produced by *L. braziliensis*-infected individuals during active disease

IL-1β is related to the severity of leishmaniasis, increasing the inflammatory response (Charmoy et al., 2016; Fernández-Figueroa et al., 2012; Novais et al., 2017). Exacerbated inflammatory responses can generate tissue damage and ulcer development in CL (Ribeiro-de-Jesus et al., 1998). To determine whether IL-1β was produced in *L. braziliensis*-infected patients, we stimulated peripheral blood mononuclear cells (PBMCs) from healthy subjects (HSs) and ECL, CL, and cured-of-CL (CCL) individuals (cured up to 5 years) with soluble *Leishmania* antigen (SLA) and assessed IL-1β levels on supernatants of these cultures by ELISA. The results show that PBMCs from *L. braziliensis*-infected patients produce IL-1β in response to SLA (Figure 1a). Moderate production of IL-1β was observed in ECL patients, and higher cytokine secretion was detected in CL, but most CCL patients did not produce IL-1β (Figure 1a). We assessed IL-1β production in CCL individuals who had been cured for up to 5 years. To investigate

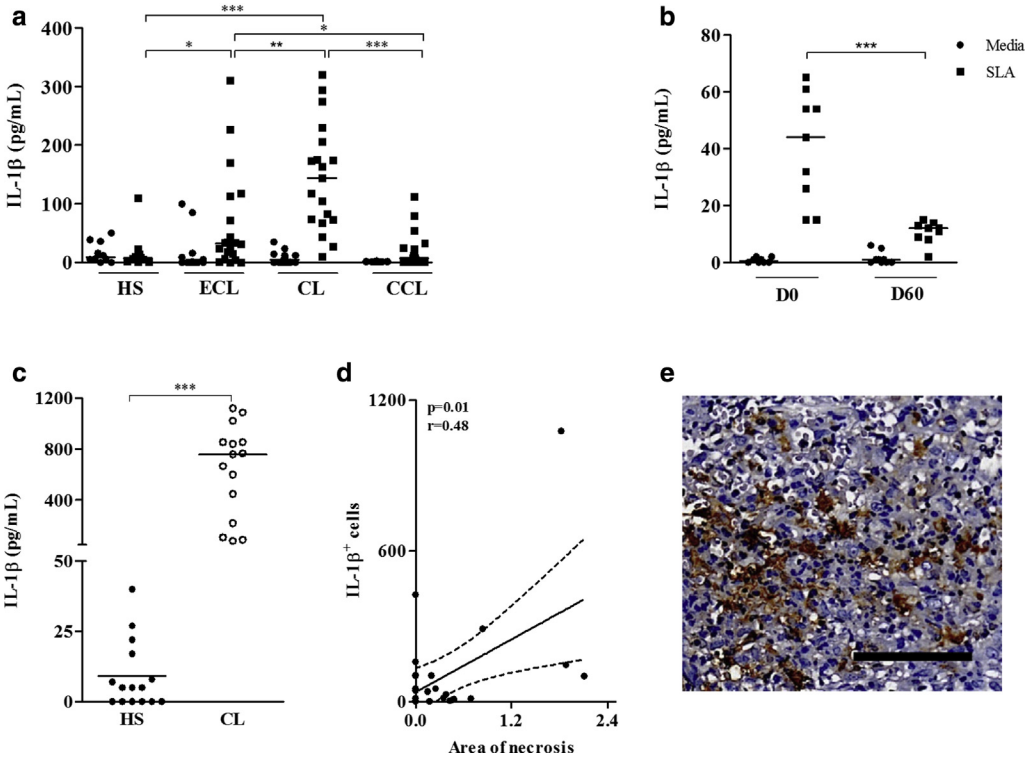

Figure 1. IL-1β is produced by *L. braziliensis*-infected individuals during active disease. IL-1β concentrations were determined by ELISA on (**a, b**) supernatants of PBMCs and (**c**) biopsy samples. (**a**) PBMCs from HSs (n = 10), ECL patients (n = 20), CL patients (n = 19), and CCL individuals (n = 19) were cultured in the presence or absence of soluble *Leishmania* antigen (SLA). (**b**) PBMCs from CL patients on days 0 and 60 after treatment were cultured or not with SLA. (**c**) Biopsy samples from healthy skin from HSs (n = 15) and lesions from CL patients (n = 15) were cultured for 72 hours. (**d**) Correlation of IL-1β$^+$ cells and areas of necrosis (%) (n = 26). (**e**) Immunohistochemistry for IL-1β was performed on lesions of CL patients. Original magnification ×20. Scale bar = 0.1 mm. Statistical analysis was performed using Mann Whitney and Pearson correlation tests. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$. CCL, cured of cutaneous leishmaniasis; CL, cutaneous leishmaniasis; D, day; ECL, early cutaneous leishmaniasis; HS, healthy subject; PBMC, peripheral blood mononuclear cell; SLA, soluble *Leishmania* antigen.

whether patients with CL would produce IL-1β right after the treatment, we measured IL-1β in the same individuals before and 60 days after the treatment had started. A significant decrease in IL-1β levels was observed after treatment (Figure 1b). In addition, with the knowledge that mononuclear cells migrate to the inflammatory site favoring inflammation, IL-1β production was assessed in lesion biopsy supernatants from CL patients. High levels of IL-1β were produced spontaneously compared with normal skin (Figure 1c). Moreover, we also determined the presence of IL-1β by immunohistochemistry and found that the presence of IL-1β correlates with areas of necrosis in CL lesions (Figure 1d and e). These data together suggest that IL-1β contributes to the pathogenesis of CL, because there was a progressive increase of this protein from the initial phase until the late stage of the disease, and after the cure, the production of IL-1β was significantly reduced.

IL-1β levels correlate with disease progression

We found that IL-1β is produced during active disease but not in cured individuals. To confirm the association IL-1β production with immunopathology, we performed correlations between the levels of IL-1β and lesion size at patient admission (Figure 2a), time since lesion started (Figure 2b), and time to heal (Figure 2c). Our results show that the time since lesion started is positively correlated with the levels of SLA-induced IL-1β. The therapeutic failure is high in CL due to *L. braziliensis*. To determine if high levels of IL-1β were associated with therapeutic failure, we performed chi-square test using the medians of ECL and CL levels of IL-1β as cutoffs for low and high IL-1β production, respectively. We found no association of high levels of IL-1β with therapeutic failure in ECL and CL PBMCs stimulated with SLA or CL biopsy samples cultured in vitro. These data indicate that IL-1β is important for ulcer appearance; however, high levels of IL-1β may not play a role in response to pentavalent antimony treatment.

NLRP3, ASC, and caspase-1 are necessary for IL-1β production in mouse macrophages

The secretion of IL-1β depends on inflammasome activation. The two inflammasome receptors mostly studied to induce IL-1β production are NLRP3 and AIM2. Both form complexes with ASC and caspase-1 for processing and secretion of IL-1β and IL-18 (Connolly and Bowie, 2014; Man et al., 2016). Because our results show that CL patients produce IL-1β and that this cytokine is associated with immunopathology, we decided to investigate in a murine model which cytosolic receptors are induced by *L. braziliensis* infection that leads to IL-1β production. We infected C57BL/6 bone marrow-derived macrophages from wild-type mice and mice deficient for NLRP3, ASC, caspase-1, AIM2, and IL-1β receptor with *L. braziliensis* and assessed on cell supernatants the levels of IL-1β production by ELISA. Our results show that IL-1β production in mice is not dependent on AIM2 inflammasome or IL-1β receptor (Figure 3). Instead, ASC and daspase-1, downstream of NLRP3 activation, is the pathway involved in IL-1β production (Figure 3). Lack of NLRP3, ASC, or caspase-1 completely abrogated IL-1β production.

Intermediate monocytes express *NLRP3*

Recent reports in mouse models of CL have shown that the production of IL-1β depends on the inflammasome NLRP3 activation, and it is known that AIM2 is also one important inflammasome receptor mediating IL-1β production (Charmoy et al., 2016; Clay et al., 2014; Man et al., 2016; Novais et al., 2017). Our in vitro results show that *L. braziliensis*-infected mouse macrophages produce IL-1β in an NLRP3-dependent manner. To assess the gene expression of NLRP3 and AIM2 in human *L. braziliensis* infection, adherent macrophages from the HSs and CL patients were stimulated with SLA, and real-time PCR was performed. We observed an increase in mRNA expression of NLRP3 in SLA-stimulated macrophages from CL patients compared with HSs and no differences in AIM2 expression between HSs and CL patients (Figure 4a). Studies have reported that circulating monocytes constitute a heterogeneous population. To determine the frequency of monocyte subsets expressing NLRP3, we assessed intracellular NLRP3 by flow cytometry (Figure 4b). Intermediate monocytes significantly expressed more NLRP3 than classical and nonclassical ones; however, upon *Leishmania* infection a decrease in the frequency of intermediate NLRP3^{+} monocytes was observed, probably due to NLRP3 inflammasome activation (Figure 4b–d). Taken together, the results show that intermediate monocytes are the main subset producing IL-1β, and we hypothesize that

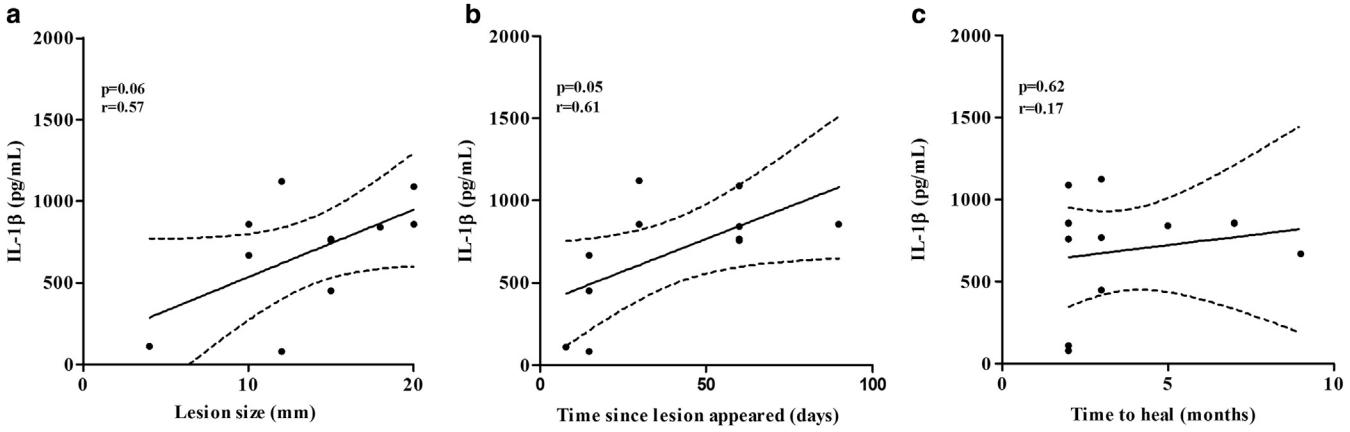

Figure 2. IL-1β levels correlate with disease progression. Correlation of IL-1β concentration on supernatants of PBMC with (**a**) lesion size, (**b**) time since lesion appearance, (**c**) and time to heal. Statistical analysis was performed using Pearson correlation test. PBMC, peripheral blood mononuclear cell.

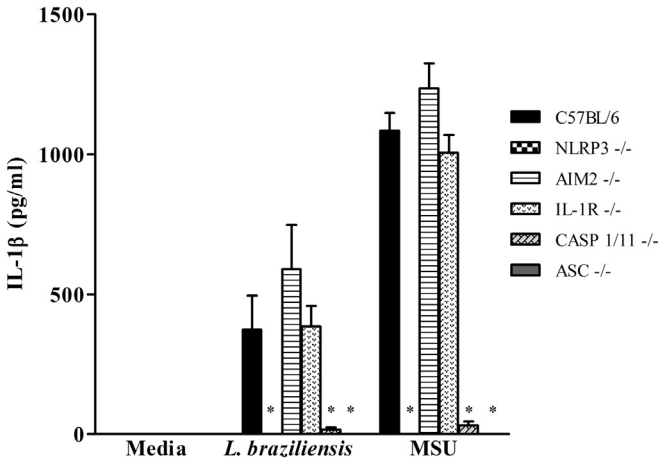

Figure 3. NLRP3, ASC, and caspase-1 are necessary for IL-1β production in mouse macrophages. BMDMs from wild-type C57BL/6 mice and mice deficient for NLRP3, ASC, caspase-1/11, AIM2, and IL-1R were prepared, pulsed with LPS (500 ng/ml), and infected with *L. braziliensis* (multiplicity of infection = 10:1) or stimulated with monosodium urate (250 μg/ml). After 48 hours of culture, ELISA for IL-1β was performed on supernatants. *$P < 0.001$, two-way analysis of variance with Bonferroni posttest, compared with wild-type C57BL/6. BMDM, bone marrow-derived macrophage; LPS, lipopolysaccharide; MSU, monosodium urate.

interaction of *Leishmania* species products with the NLRP3 inflammasome decreases its availability.

Intermediate monocytes are the main source of IL-1β

We recently documented that intermediate monocyte frequency is increased during CL and that these cells are the main source of TNF. To test the hypothesis that intermediate monocytes are the main cell producing IL-1β, we cultured PBMCs from HSs in the presence of SLA and performed intracellular staining for IL-1β. Our results show that intermediate monocytes are the main source of IL-1β in response to SLA (Figure 5).

Phagocytosis and killing of *Leishmania* species does not depend on presence of IL-1β

To investigate whether IL-1β plays any role on parasite killing in CL, we infected human macrophages in the presence or absence of anti-IL-1β neutralizing antibodies or recombinant IL-1β and assessed parasite counts by microscopy in different time points after infection. As we have reported before (Giudice et al., 2012), macrophages from humans are able to control *Leishmania* species growth in vitro in the absence of any stimuli (Figure 6). Neither blockade nor addition of recombinant IL-1β had any influence on parasite counts within macrophages (Figure 6). This shows no participation of IL-1β in *Leishmania* species killing within human macrophages, suggesting that blockade of the IL-1β pathway would benefit the patient without any risk of increasing parasite growth.

DISCUSSION

Tissue damage caused by exacerbated inflammatory response to *L. braziliensis* leads to skin ulcer development. Studies in humans and experimental models of CL indicate that TNF and IL-1β are important cytokines mediating inflammation in these patients. Studies to determine cell phenotypes inducing tissue damage and ulcer development in CL have mainly focused on the role of T lymphocytes, and several studies have shown a protection function for $CD4^+$ T cells and a deleterious role for $CD8^+$ T cells (Cardoso et al., 2015; Novais et al., 2017; da Silva Santos et al., 2013). Circulating monocytes are heterogeneous, and we previously showed that intermediate monocytes ($CD14^+CD16^+$) are the main source of TNF in CL patients, suggesting that these cells may contribute to immunopathology (Passos et al., 2015). In this work we found that intermediate monocytes are the major source of IL-1β, suggesting that these cells and also IL-1β could be therapeutic targets.

The presence of IL-1β, gene expression or protein, in lesions of CL patients infected by *L. mexicana* and *L. braziliensis* called attention to a possible deleterious activity for this cytokine in CL (Fernández-Figueroa et al., 2012; Novais et al., 2015). By assessing mRNA expression in lesions of CL patients, we showed that shortly after infection the inflammatory pathways are activated in the skin, favoring the appearance of the ulcer in CL (Novais et al., 2015). Here, we showed that from the early stage of the disease there is a progressive increase in the production of IL-1β and that after cure there is a significant reduction in IL-1β levels, suggesting that the presence of this cytokine is associated with pathology rather than protection. These data are in concordance with our previous work showing that upon cure a decrease in the production of the inflammatory cytokines IFN-γ and TNF is observed (Carvalho et al., 2013). Another result that emphasizes the hypothesis that IL-1β participates in the pathogenesis of CL is the positive correlation between IL-1β–positive cells with areas of necrosis, found here in the lesions of CL patients.

IL-1β is produced mainly by monocytes in the blood, and several recent reports show biological heterogeneity in the phenotype and function of these cells, in which the intermediate monocytes have a more inflammatory profile than classical and nonclassical subsets (Dinarello, 2009; Dinarello et al., 2012; Guo et al., 2015; Netea et al., 2009; Roerink et al., 2017; Zawada et al., 2011; Ziegler-Heitbrock, 2015; Ziegler-Heitbrock et al., 2010). This characteristic has already been addressed by our group in CL patients, in whom we found that intermediate monocytes are increased in frequency soon after infection, have greater potential for migration to lesions, and produce more TNF, increasing inflammation (Passos et al., 2015). Here we have expanded those studies and shown that the main source of IL-1β is the intermediate monocytes, further showing an important role of this cell in promoting inflammation in *L. braziliensis* infection.

The maturation and release of IL-1β by monocytes involves processing of pro–IL-1β, which can be dependent on inflammasome activation. The NLRP3 inflammasome leads to ASC activation and caspase-1 processing, both necessary for IL-1β production. Previous studies have concluded that the NLRP3 pathway is the main route of IL-1β production promoting pathology (Gurung et al., 2015; Novais et al., 2015, 2017). We have recently documented that $CD8^+$ T-cell–induced pathology depends on NLRP3 signaling that is required for maintenance of elevated IL-1β levels in patient lesions (Novais et al., 2017). The use of a nonhealing strain of *L. major* also showed that NLRP3 inflammasome-dependent

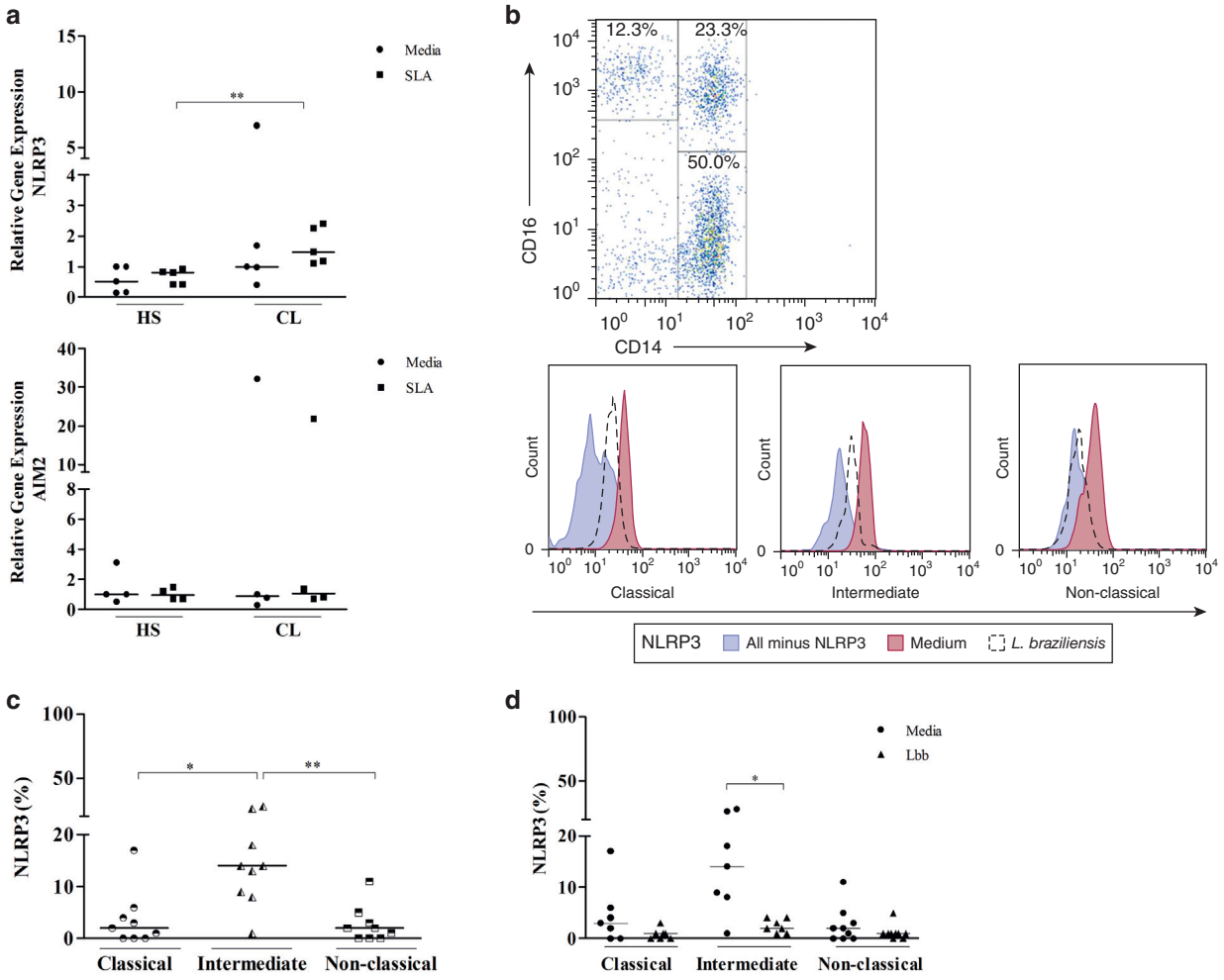

Figure 4. Intermediate monocytes express NLRP3. (**a**) NLRP3 and AIM2 gene expression, represented as $2^{-\Delta\Delta CT}$, after real-time PCR of RNA from macrophages-derived monocytes of HSs (n = 5) and CL patients (n = 5) stimulated or not with SLA for 2 hours. (**b**) Gating strategy to assess monocyte subsets based on size and complexity, followed by CD14 and CD16 expression and histograms representative of NLRP3 expression in infected monocytes. The gate strategy was done based on all −1 staining. (**c**) Frequency of NLRP3 ex vivo expression was determined by intracellular staining in monocyte subsets. (**d**) Monocytes from CL patients were infected or not with *L. braziliensis* (ratio 5:1) and labeled for CD14, CD16, and NLRP3. Statistical analyses were performed using the Mann-Whitney test and the Wilcoxon rank test. $^*P < 0.05$, $^{**}P < 0.01$. CL, cutaneous leishmaniasis; HS, healthy subject; SLA, soluble *Leishmania* antigen.

IL-1β plays a crucial role in the development of nonhealing cutaneous leishmaniasis in conventionally resistant mice (Charmoy et al., 2016). In view of the importance of this IL-1β production pathway in leishmaniasis, we decided to evaluate the expression of NLRP3 in CL patients and observed that the gene expression in patients' macrophages was higher than in healthy individuals and that the intermediate monocytes had higher NLRP3 protein expression than other monocyte subsets that decreased shortly after infection with *L. braziliensis*. These data argue in favor of the idea that the pathway induced by *Leishmania* species in human mononuclear phagocytes is also through NLRP3 activation. The molecule from *Leishmania* species activating NLRP3 still needs to be discovered; however, it does not seem to be the most abundant surface molecule from *Leishmania* species, GP63, because a recent study showed that GP63 can significantly inhibit NLRP3 activation (Shio et al., 2015). AIM2 is another important inflammasome that can trigger IL-1β production and inflammation. Although it was recently shown to increase expression of AIM2 in CL lesions (Moreira et al., 2017; Novais et al., 2015), two of our results indicate that IL-1β production does not depend on AIM2 activation in CL: first, we found low expression of AIM2 in CL patients' macrophages, even in *Leishmania* species antigen-stimulated cultures; second, lack of AIM2 did not have any effect on IL-1β production in mice macrophages.

Altogether, our results strongly imply a deleterious role for IL-1β in CL and point out intermediate monocytes as the main cell type producing this cytokine, thus participating in the pathologic process observed in CL patients. Drugs to inhibit IL-1β production have been used in vivo in mouse models of CL, ameliorating symptoms of the disease. We also previously showed that the use an NLRP3 inhibitor reduced IL-1β production in biopsy samples from CL patients (Novais et al., 2017). Because IL-1β does not seem to participate in the control of *Leishmania* parasites in humans, the use of drugs that block IL-1β production in combination with antiparasitic drugs may be helpful as an immunotherapy in CL.

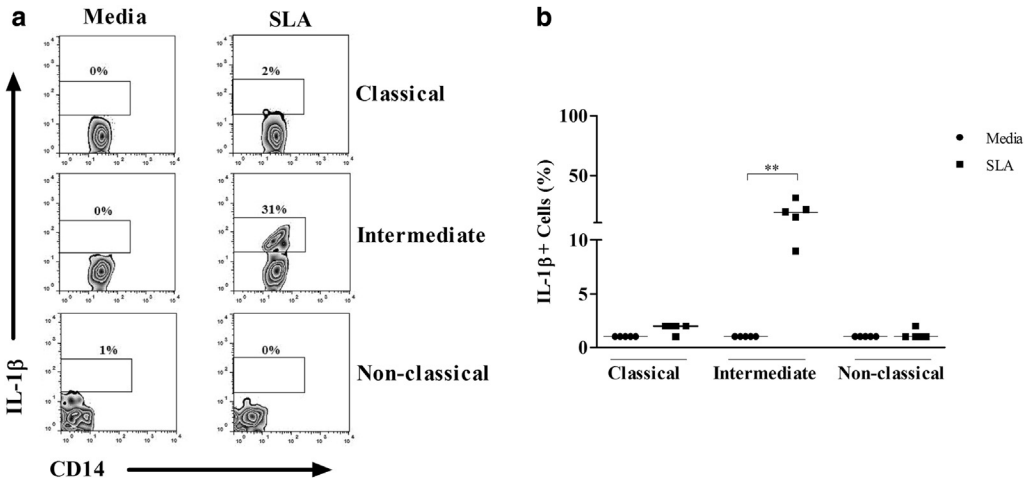

Figure 5. Intermediate monocytes are the main source of IL-1β. PBMCs were obtained from HSs (n = 5), and cultured in the presence or absence of SLA for 8 hours in the presence of Golgi Stop (BD Biosciences, Franklin Lakes, NJ). Staining for CD14, CD16, and the frequency of monocyte subsets producing IL-1β was determined by intracellular staining. (**a**) Representative plots showing frequencies of monocyte subsets producing IL-1β. (**b**) Frequency of IL-1β–producing cells from each monocyte subset. Statistical analysis was performed using the Wilcoxon rank test, and results were considered significant at $^{**}P < 0.005$. PBMC, peripheral blood mononuclear cell; SLA, soluble *Leishmania* antigen.

MATERIALS AND METHODS

Ethical statement

This study was approved by the Ethics and Research Committee from Federal University of Bahia (25/12) and the National Commission of Ethics in Research (612.907). All individuals were adult volunteers who provided written informed consent. This work was conducted in accordance with the Declaration of Helsinki.

Animal experiments were approved by the Institutional Animal Care and Use Committee of Universidade Federal de Minas Gerais (Ethics Committee for Animal Experimentation [CETEA] #128/2014). All animal experiments were conducted in accordance with Brazilian Federal Law number 11.794, which regulates the scientific use of animals, and Institutional Animal Care and Use Committee guidelines.

Subjects

The sample was composed of 20 individuals with ECL, 35 with CL, and 19 CCL individuals from the *L. braziliensis* transmission area of Corte de Pedra, Bahia, Brazil and 15 HSs living in an area where *Leishmania* species are not endemic. ECL patients were characterized by the presence of a lymphadenopathy or lymphadenopathy accompanied by a papule or an exoulcerative lesion and a positive PCR result. Diagnosis of CL was made based on the presence of typical skin ulcer associated with a positive PCR result, as previously described (Machado et al., 2010; Weirather et al., 2011). The group of CCL individuals was composed of individuals without active disease and up to 5 years after cure. The *Leishmania* species skin test was done in all patients, and the immunological analyses were performed before therapy or up to 5 years after therapy.

Parasite culture

Isolate of *L. braziliensis* (MHOM/BR/LTCP11245) was obtained from a skin lesion of a CL patient and identified as *L. braziliensis* by multilocus enzyme electrophoresis (Cupolillo et al., 1994). The parasites selected for this study had not been previously passaged in liquid culture medium. After selection, the parasites were expanded in Schneider's medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Waltham, MA), 2% sterile human urine and gentamicin (50 μg/ml) (Gibco) (Grekov et al., 2011).

Soluble *Leishmania* antigen

SLA was prepared with an isolate of *L. braziliensis* as previously described (Reed et al., 1986). Briefly, promastigotes resuspended in lysis solution (Tris, HCl, EDTA, and leupeptin) were immersed in liquid nitrogen and thawed at 37°C. After freezer-thaw procedure, they were sonicated, and the disrupted parasites were centrifuged at 14,000*g*. The supernatant was filtered and assayed for protein

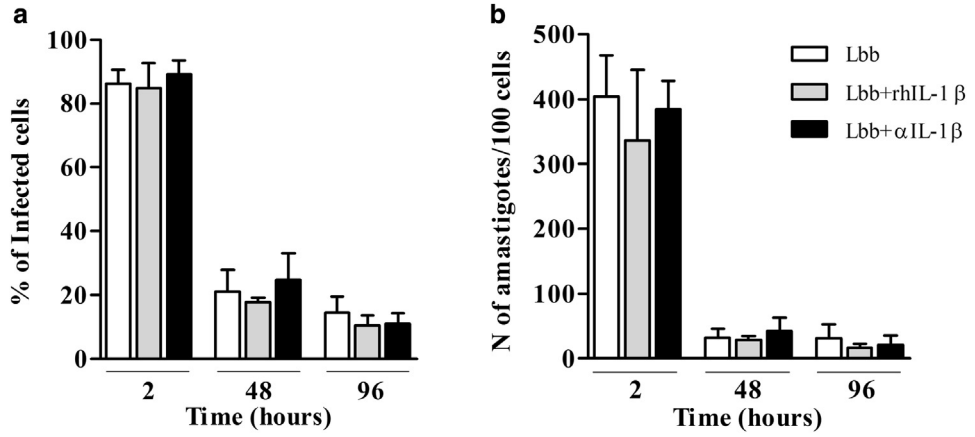

Figure 6. Phagocytosis and killing of *Leishmania* does not depend on presence of IL-1β. Monocyte-derived macrophages from HS (n = 5) were infected with *L. braziliensis* in the stationary phase (ratio 5:1), stimulated with recombinant (rhIL-1β) (20 ng/ml) or anti-IL-1β (5 μg/ml) and cultured for 2, 48, and 96 hours. (**a**) Frequency of infected cells at different time points. (**b**) Number of *Leishmania* amastigotes/100 macrophages. Data represent the mean ± standard deviation. HS, healthy subject.

concentration, tested for endotoxin using the *Limulus* amebocyte lysate test, and used at a concentration of 5μg/ml.

Culture of PBMCs and biopsy samples

PBMCs were isolated from heparinized venous blood by Ficoll-Paque (GE Healthcare) gradient centrifugation and after washing steps in saline, the cell concentration was adjusted to 3×10^6 cells in 1 ml of RPMI-1640 (Gibco) supplemented with 10% FBS (Gibco), 100 U penicillin/ml, and 100 μg streptomycin/ml. PBMCs were dispensed into 24-well plates and incubated at 37°C, 5% CO_2 for 72 hours in the presence or absence of 5 μg/ml SLA.

Biopsy samples from *L. braziliensis* patients and HSs were performed using a 4-mm punch and were cultured in complete RPMI media without stimuli at 37°C, 5% CO_2 for 72 hours. Supernatants of PBMCs and biopsy samples were collected and stored at −70°C for analysis of IL-1β by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The results are expressed in pg/ml.

Flow cytometry

Flow cytometry was performed as previously described (Sornasse et al., 1996). Briefly, PBMCs (1×10^6) were stimulated with SLA or exposed to promastigotes of *L. braziliensis* at a ratio of 5:1 cells at 37°C in 5% CO_2. After 2 hours, extracellular parasites were removed after centrifugation, and cells were incubated for additional 8 hours in the presence BD Golgi Stop Protein Transport Inhibitor (BD Biosciences, Franklin Lakes, NJ). For ex vivo cell surface staining, incubated cells with monoclonal antibodies anti-CD14 and anti-CD16 (BD Pharmingen, San Diego, CA) or (eBioscience, San Diego, CA), for 15 minutes, washed by centrifugation twice, and fixed with 2% paraformaldehyde. For intracellular staining, cells were resuspended in Perm/Wash (BD Biosciences) for 15 minutes, and intracellular labeling was performed using monoclonal antibody anti-IL-1β (eBioscience) and anti-NLRP3 (R&D Systems) for 30 minutes.

Human macrophage cultures

Monocyte-derived macrophages were prepared following a method previously shown (Giudice et al., 2012) by our laboratory to yield 99% macrophages characterized by flow cytometry as CD14-positive, CD3-negative, CD19-negative. Briefly, peripheral blood mononuclear cells were separated from blood over Ficoll hypaque (GE Healthcare, Chicago, IL), and monocytes were separated by adherence to plastic. Cells were maintained in Teflon vials in RPMI-1640 (Gibco) supplemented with 10% FBS (Gibco), 100 U penicillin/ml, and 100 μg streptomycin/ml. After six days of culture at 37°C and 5% CO_2, differentiated macrophages were harvested by centrifugation, resuspended, and allowed to adhere to glass coverslips for 24 hours at 37°C and 5% CO_2. After this incubation period, cells were stimulated or not with SLA or infected with *L. braziliensis* and stimulated with 20 ng/ml recombinant IL-1β (rhIL-1β) (Invitrogen, Waltham, MA) or 5 μg/ml anti-IL-1β (R&D Systems); cultivated in RPMI-1640 (Gibco); supplemented with 10% FBS (Gibco), 100 U penicillin/ml, and 100 μg streptomycin/ml; and incubated at 37°C, 5% CO_2 for 2, 48 or 96 hours. After each time point, the infection rate and the parasite burden were evaluated by microscopy.

RNA extraction and *NLRP3* and *AIM2* gene expression

Cells stimulated or not with SLA and incubated at 37°C, 5% CO_2 for 2 hours were harvested in TRIzol Reagent (Invitrogen). RNA was extracted by using the PureLink RNA Mini Kit (Ambion, Waltham, MA) according to the manufacturer's instructions. RNA concentration and integrity were determined by spectrophotometric optical density measurement (260 and 280 nm). The analysis of gene expression was performed as previously described (Almeida et al., 2017).

Immunohistochemistry

Tissues obtained from 26 skin biopsy samples of patient CL lesions were fixed in buffered formaldehyde and embedded in paraffin. Deparaffinization and rehydration of 5-μm–thick sections were performed using xylene and absolute alcohol and antigen retrieval, using citrate buffer pH 6.0 at 96°C for 20 minutes. Immunohistochemistry reactions were performed as previously described by Saldanha et al. (2017). Briefly, after blockage of peroxidase activity with 3% hydrogen peroxide for 10 minutes and proteins with Protein Block Serum-Free (DAKO, Carpinteria, CA) for 15 minutes. The slides were incubated overnight at 4°C with Monoclonal Mouse IL-1β (Cell Signaling Technology, Danvers, MA). Mouse and Rabbit Peroxidase Kit/Horseradish Peroxidase KP500 (Diagnostic BioSystems, Pleasanton, CA) were used to perform the reaction according to the manufacturer's recommendations.

Mouse macrophage cultures and infection

Wild-type C57BL/6 mice and genetically deficient mice for $AIM2^{-/-}$, $ASC^{-/-}$, caspase-1/$11^{-/-}$, $NLRP3^{-/-}$, and $IL\text{-}1R^{-/-}$ were previously described (Kuida et al., 1995; Lara-Tejero et al., 2006; Rathinam et al., 2010; Vandanmagsar et al., 2011). The mice were maintained at Universidade Federal de Minas Gerais and used at 6-8 weeks of age. Bone marrow-derived macrophages were prepared and infected as previously described (Lima-Junior et al., 2013; Marim et al., 2010). Briefly, bone marrow cells were isolated from the femurs and tibias of the animals and cultured in RPMI 1640 supplemented with 30% L929 cell-conditioned medium and 20% FBS for 7 days. Overall, 0.5×10^6 bone marrow-derived macrophages were treated or not with lipopolysaccharide for 6 hours (500 ng/ml) and stimulated with monosodium urate (250 μg/ml) or infected with stationary phase *Leishmania braziliensis* (multiplicity of infection = 10:1) for 42 hours. After 48 hours the supernatants were harvested, and IL-1β concentrations were detected by ELISA.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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