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IL-1β production by intermediate monocytes is associated with immunopathology in cutaneous leishmaniasis

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Abstract

Cutaneous leishmaniasis due to *Leishmania braziliensis* infection is an inflammatory disease which skin ulcer development is associated with mononuclear cells infiltrate and high levels of inflammatory cytokines production. Recently, NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome activation and IL-1β production has 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 work we show high production of IL-1β in biopsies and *Leishmania* antigen-stimulated peripheral blood mononuclear cells (PBMC) 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*

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CONFLICT OF INTEREST

The authors state no conflict of interest.

was dependent on NLRP3, Caspase-1 and caspase-recruiting domain (ASC). Additionally, we observed an increased expression of NLRP3 gene in macrophages and NLRP3 protein in intermediate monocytes from cutaneous leishmaniasis patients. These results identify an important role for human intermediate monocytes for the production of IL-1β which contributes to the immunopathology observed in cutaneous leishmanisis patients.

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 non-ulcerated 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, Carvalho et al., 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 site, whereas CD4+ T cells are associated with protection (Cardoso et al., 2015, Novais et al., 2017, Santos Cda et al., 2013).

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 non-classical (CD14+CD16++) subsets (Almeida et al., 2017, Zawada et al., 2011, Ziegler-Heitbrock, 2015, Ziegler-Heitbrock et al., 2010). Monocyte subsets do not only differ 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 non-classical) are increased in the peripheral blood of *L. braziliensis*-infected individuals (Passos et al., 2015). Our data also shows that intermediate monocytes were the main source of TNF, an inflammatory cytokine in which the levels are increased during CL and highly associated with tissue damage and lesion development (Passos et al., 2015). These data argues 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, Dinarello et al., 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 not on inflammasome activation to become biologically active (Martinon et al., 2002, Netea et al., 2009). The formation of inflammasome

complexes is an important pro-inflammatory pathway of the innate immunity (Clay et al., 2014). The NLRP3 inflammasome is the best-studied with the list of agonists, as low intracellular K^+ concentration, extracellular ATP, 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 demonstrated that NLRP3 is directly involved in processing and releasing of IL-1β during *Leishmania* infection 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 inflammatory process in leishmaniasis, exacerbating disease (Charmoy et al., 2016, Novais et al., 2017).

In this manuscript we documented that intermediate monocytes from CL patients secrete IL-1β and express NLRP3, and that IL-1β production increase with CL progression. Further, the amount of IL-1β positively correlates with areas of necrosis and time of lesion in CL patients. Moreover, our results in mice showed 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 (Ribeirode-Jesus et al., 1998). To determine whether IL-1β was produced in *L. braziliensis*-infected patients, we stimulated PBMC from healty subjects (HS), 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 PBMC 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 that had cured up to 5 years. To investigate 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 when compared to 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 1 d and e). These data together suggest that IL-1β contributes to the pathogenesis of CL, since 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 correlates 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 was associated with therapeutic failure we performed chi-square test using the medians of ECL and CL levels of IL-1β as a cutoff for low and high IL-1β production. We found no association of high levels of IL-1β with therapeutic failure in ECL and CL PBMC stimulated with SLA or CL biopsies 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 complex with ASC and Caspase-1 for processing and secretion of IL-1β and IL-18 (Connolly and Bowie, 2014, Man et al., 2016). Since our results show that CL patients produce IL-1β and this cytokine is associated with immunopathology, we decided to investigate in 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 (BMDM) from WT 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 Caspase-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 infammasome NLRP3 activation and its 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 NLRP3-dependent manner. To assess the gene expression of NLRP3 and AIM2 in human *L. braziliensis* infection, adherent macrophages from the HS and CL patients were stimulated with SLA and Real-Time PCR was performed. We observed an increase in mRNA expression NLRP3 in SLA-stimulated macrophages from CL patients when compared to HS and no differences on AIM2 expression between HS and CL (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 non-classical ones; however, upon *Leishmania* infection a decrease in the frequency of

intermediate monocytes NLRP3^{+} was observed, probably due to NLRP3 inflammasome activation (Figure 4 b, c and d). Taken together, the results show that intermediate monocytes are the main subset producing IL-1β and hypothesize that interaction of *Leishmania* products with NLRP3 inflammasome decreases its availability.

Intermediate monocytes are the main source of IL-1β

We have recently documented that intermediate monocytes 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 PBMC from HS 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* 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 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 published before (Giudice et al., 2012), macrophages from human are able to control *Leishmania* 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* killing within human macrophages, suggesting that blockade of 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 human 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, Santos Cda et al., 2013). Circulating monocytes are heterogeneous and we have previously shown 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 the present 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 lesion of CL patients infected by *L. mexicana* and *L. braziliensis* called attention for a possible deleterious activity for this cytokine in CL (Fernández-Figueroa et al., 2012, Novais et al., 2015). By assessing mRNA expression in lesion 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 demonstrated that from the early stage of the disease there is a progressive increase in the production of IL-1β and after cure, 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 that shows 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 non-classical 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, where 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 demonstrated that the main source of IL-1β is the intermediate monocytes, further demonstrating 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 that 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., 2017, Novais et al., 2015). 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 non-healing strain of *L. major* also revealed that NLRP3 inflammasome-dependent IL-1β plays a crucial role in the development of non-healing 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, the intermediate monocytes had higher NLRP3 protein expression than other monocyte subsets that decreased shortly after infection with *L. braziliensis*. These data argues in favor that the pathway induced by *Leishmania* in human mononuclear phagocytes is also through NLRP3 activation. The molecule from *Leishmania* activating NLRP3 still needs to be discovered, however, it does not seems to be the most abundant surface molecule from *Leishmania*, glycoprotein 63 (GP63), since 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 increased 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* antigens 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 points out intermediate monocyte 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 model of CL, ameliorating symptoms of the disease. We also previously showed that the use an NLRP3 inhibitor reduced IL-1β production biopsies from CL patients (Novais et al., 2017). As IL-1β does not seem to participate in the control of *Leshmania* 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.

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 volunteers adult and provided written informed consent. This work was conducted in according with the Declaration of Helsinki.

Animal experiments were approved by the Institutional Animal Care and Use Committee of Universidade Federal de Minas Gerais (UFMG) (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 IACUC guidelines.

Subjects

The sample was composed by 20 individuals with ECL, 35 CL and 19 CCL from the *L. braziliensis* transmission area of Corte de Pedra, Bahia, Brazil, and 15 HS living in non-endemic area of *Leishmania*. ECL patients were characterized by the presence of a lymphadenopathy or lymphadenopathy accompanied by a papule or an exoulcerative lesion and a positive PCR. Diagnosis of CL was done based on the presence of typical skin ulcer associated with a positive PCR, as previously described (Machado et al., 2010, Weirather et al., 2011). The group of CCL was composed by individuals without active disease and with up to 5 years after cure. The *Leishmania* skin test was done in all patients and the immunological analyses was performed prior to therapy or up to 5 years after therapy.

Parasite culture

Isolate of *L. braziliensis* (MHOM/BR/LTCP11245) was obtained from a skin lesion of 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) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 2% sterile human urine and gentamicin (50 μg/ml) (Gibco) (Grekov et al., 2011).

Soluble *Leishmania* Antigen (SLA)

SLA was prepared with an isolate of *L. braziliensis* as previously described (Reed et al., 1986). Briefly, promastigotes ressuspended 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 14,000G. The

supernatant was filtered and assayed for protein concentration, tested for endotoxin using the Limulus amebocyte lysate test and used at a concentration of 5μg/ml.

Culture of peripheral blood mononuclear cells and biopsies

PBMC 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. PBMC were dispensed into 24-well plates and incubed at 37°C 5% CO_2 for 72 hours in the presence or absence of 5 μg/ml SLA.

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

Flow cytometry

Flow cytometry was performed as previously described (Sornasse et al., 1996). Briefly, PBMC (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 hour, extracellular parasites were removed after centrifugation and cells incubated for additional 8 hours in presence BD Golgi Stop Protein Transport Inhibitor (BD Biosciences). For *ex-vivo* cell surface staining, incubated cells with monoclonal antibodies anti-CD14 (APC) and anti-CD16 (PE) (BD Pharmingen) or (FITC) (eBioscience), for 15 minutes, washed by centrifugation twice and fixed with 2% paraformaldehyde. For intracellular staining, cells were ressuspended in Perm/Wash (BD Biosciences) for 15 minutes and intracellular labeling was performed using monoclonal antibody anti-IL-1β (FITC) (eBioscience) and anti-NLRP3 (PE) (R&D Systems) for 30 minutes.

Human macrophages 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), and monocytes 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 culture at 37°C and 5% CO_2, differentiated macrophages were harvested by centrifugation, ressuspended and allowed to adhere to glass coverslips for 24 h at 37°C and 5% CO_2. Following 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) or 5μg/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, 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) according to the manufactures instructions. RNA concentration and integrity were determined by spectrophotometric optical density measurement (260 and 280 nm). The analysis of the gene expression was performed as previously described (Almeida et al., 2017).

Immunohistochemistry

Tissues obtained from 26 skin biopsies of CL lesions patients were fixed in buffered formaldehyde and embedded in paraffin. Deparaffinization and rehydration of 5-μm thick sections were performed using xylene and alcohol PA 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) for 15 minutes. The slides were incubated overnight at 4°C with Monoclonal Mouse IL-1 beta (Cell Signaling Technology). Mouse and Rabbit Peroxidase Kit/ Horseradish Peroxidase KP500 (Diagnostic BioSystems) 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-$1/11^{-/-}$, $NLRP3^{-/-}$, IL-$1R^{-/-}$ 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 UFMG and used at 6–8 weeks of age. Bone marrow derived macrophages (BMDM) 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, cultured in RPMI 1640 supplemented with 30% L929 cell-conditioned medium and 20% FBS for 7 days. 0.5×10^6 BMDM were treated or not with lipopolysaccharide (LPS) for 6 hours (500 ng/ml) and stimulated with monosodium urate (MSU) (250 μg/ml) or infected with stationary phase *Leishmania braziliensis* (MOI 10:1) for 42 hours. After 48 hours the supernatants were harvested and IL-1β concentration were detected by ELISA.

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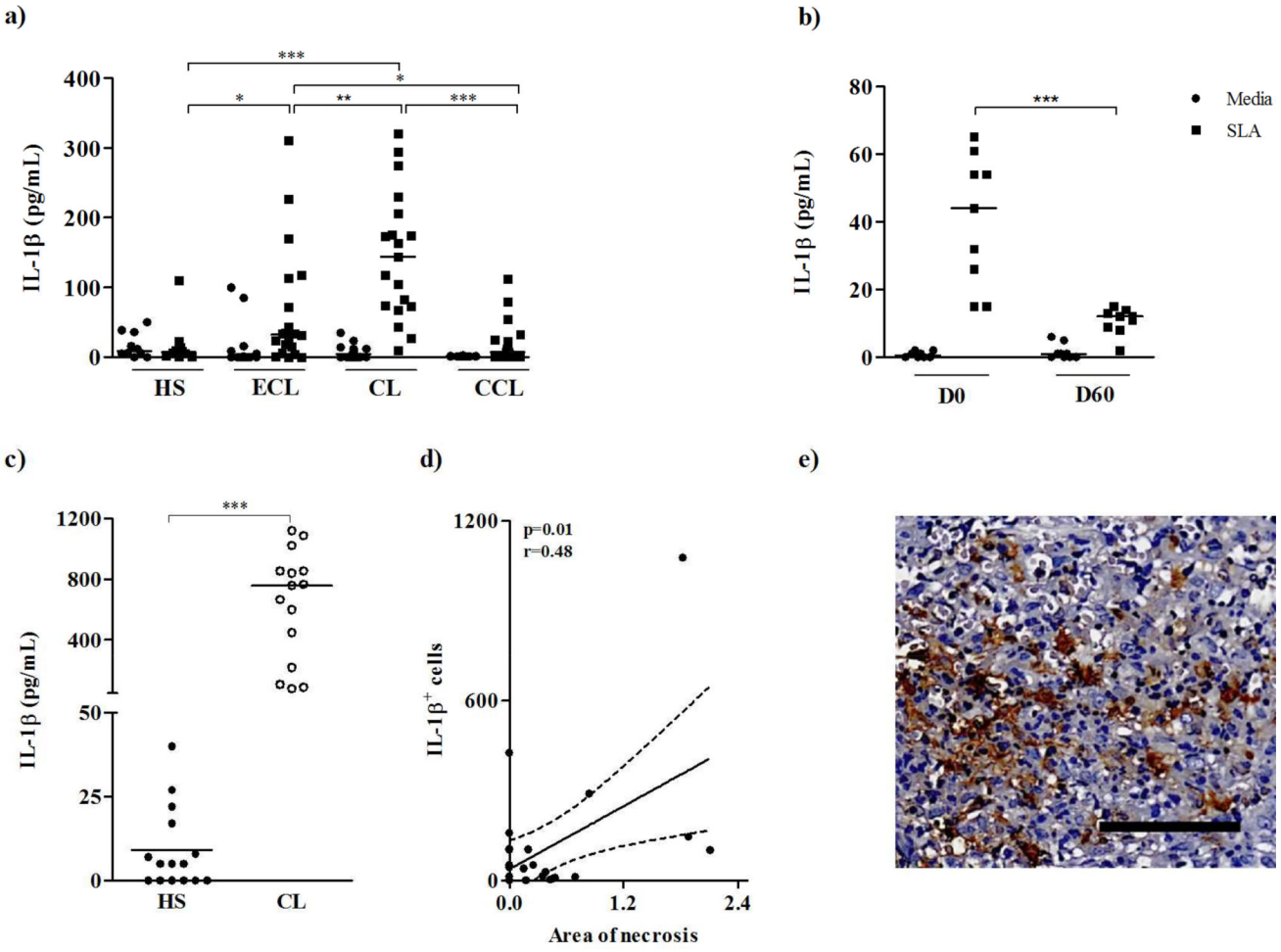

Figure 1. IL-1β is produced by *L. braziliensis*-infected individuals during active disease

IL-1β concentrations were determined by ELISA on supernatants of PBMC (a and b) and biopsies (c). (a) PBMC from HS (N=10), ECL (N=20), CL (N=19) and CCL (N=19) were cultured in the presence or absence of soluble *Leishmania* antigen (SLA). (b) PBMC from CL on days 0 and 60 post treatment were cultured with or not SLA. (c) Biopsies from healthy skin from HS (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 lesion of CL patients (20X). Scale bar = 0.1 mm. Statistical analysis was performed using Manny Whitney and Pearson correlation tests. * p<0.05, ** p<0.001; ***p<0.0001.

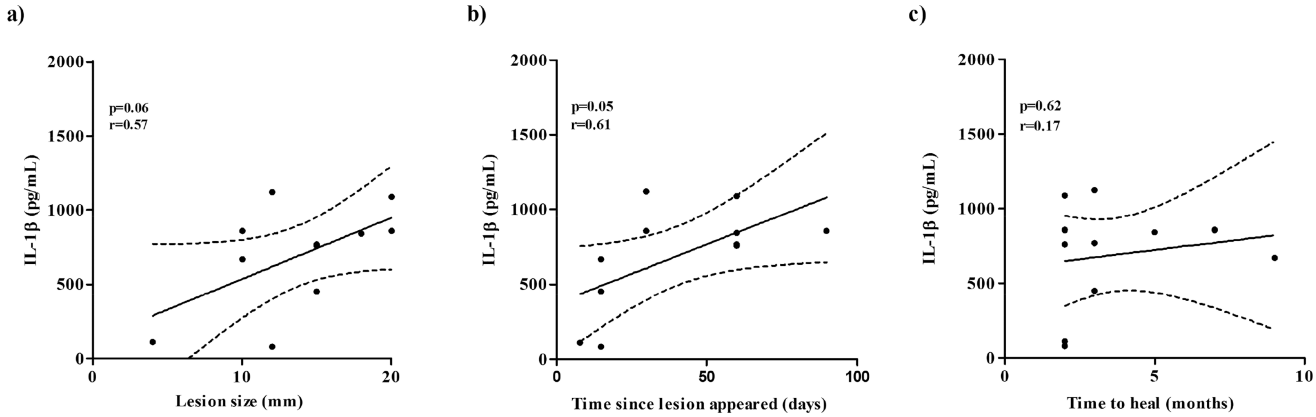

Figure 2. IL-1β levels correlates with disease progression

Correlation of IL-1β concentration on supernatants of PBMC with lesion size (a), time since lesion appearance (b) and time to heal (c). Statistical analysis was performed using Pearson correlation test.

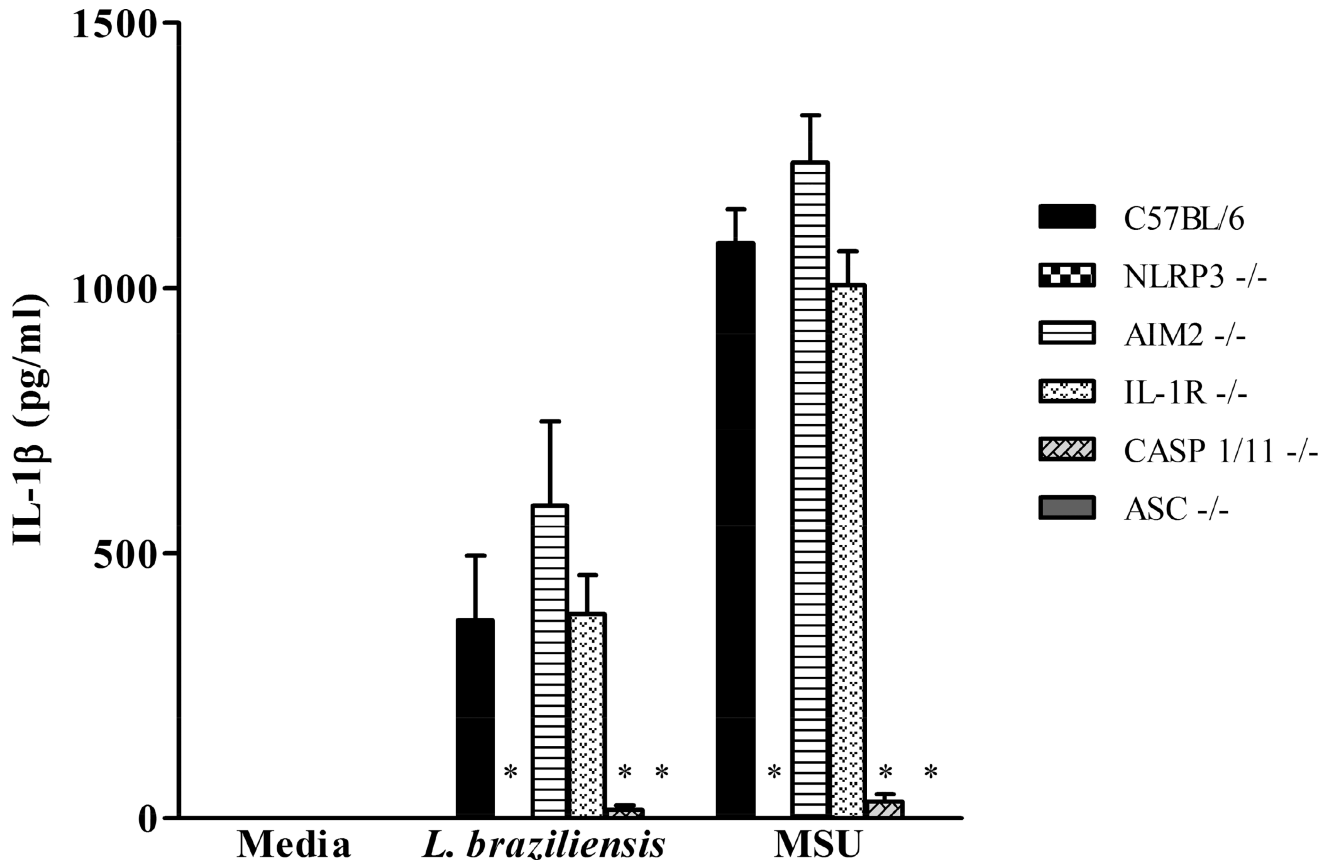

Figure 3. NLRP3, ASC and Caspase-1 are necessary for IL-1β production in mouse macrophages

BMDMs from wild-type C57BL/6 mices and deficient for NLRP3, ASC, Caspase-1/11, AIM2 and IL-1R were prepared, pulsed with LPS (500 ng/ml) and infected with *L. braziliensis* (MOI 10:1) or stimulated with monosodium urate (MSU) (250 μg/ml). After 48 hours of culture ELISA for IL-1β was performed on supernatants. * $p<0.001$, two-way ANOVA with Bonferroni's posttest, compared with wild-type C57BL/6.

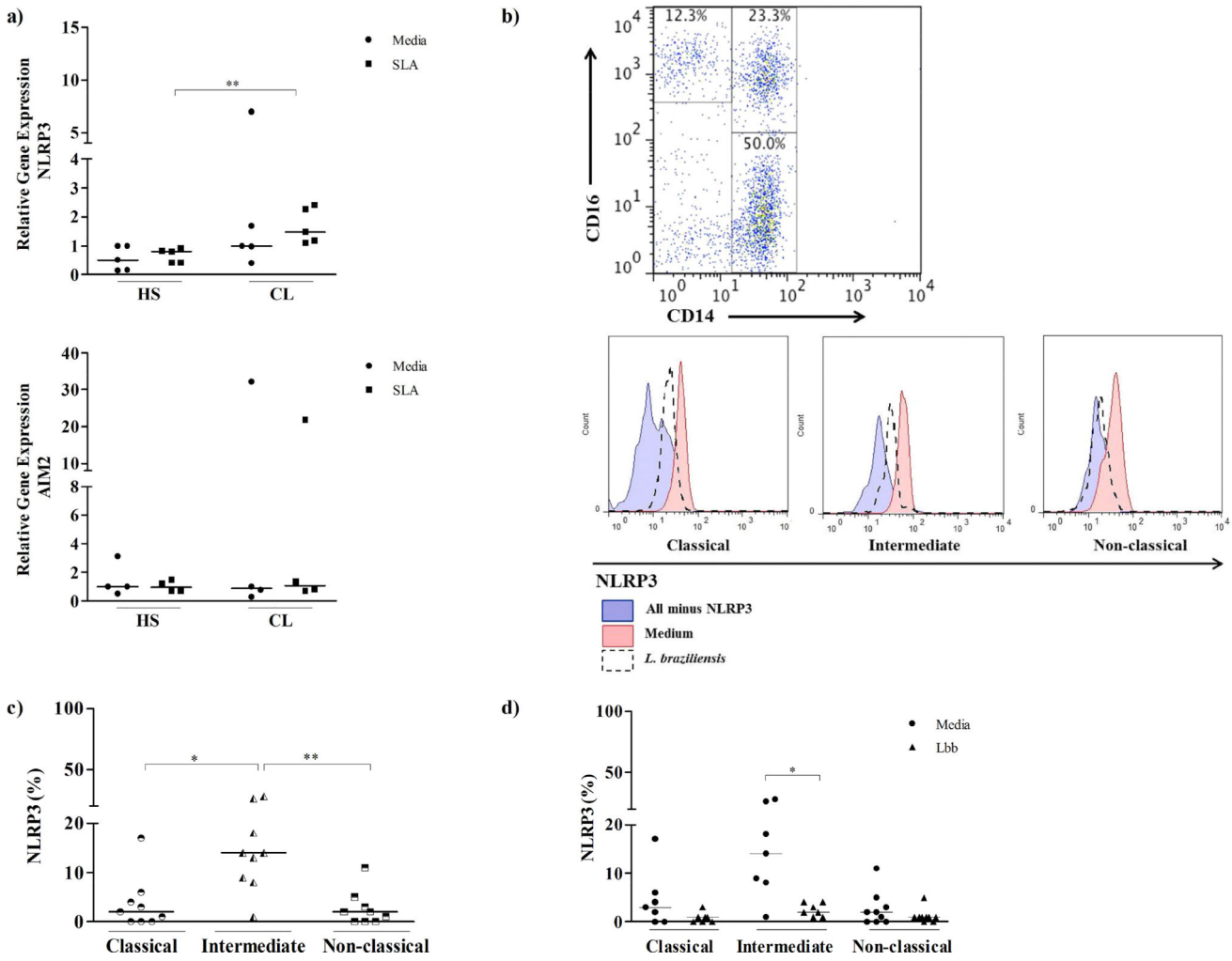

Figure 4. Intermediate monocytes express NLRP3

(a) NLRP3 and AIM2 gene expression, represented as $2^{-\Delta\Delta CT}$, following RT-PCR of RNA from macrophages-derived monocytes of HS (N=5) and CL (N=5) stimulated or not with SLA for 2 hours. (b) Gating strategy to assess monocyte subsets based on size and complexicity, followed by CD14 and CD16 expression and histograms representative of NLRP3 expression in infected monocytes. The gate strategy was done based on all minus one 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. * $p<0.05$, ** $p<0.01$.

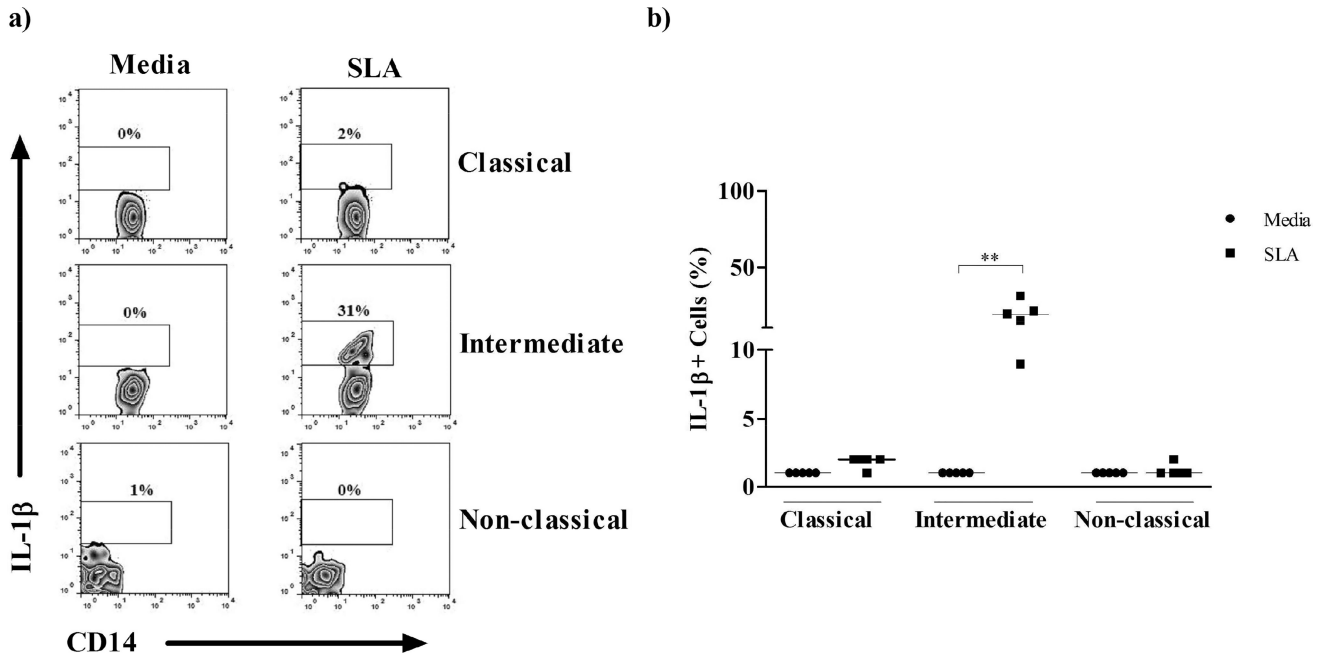

Figure 5. Intermediate monocytes are the main source of IL-1β

PBMC were obtained from HS (N =5), and cultured in presence or absence of SLA for 8 hours in the presence of Golgi Stop. 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 with a **p< 0.005.

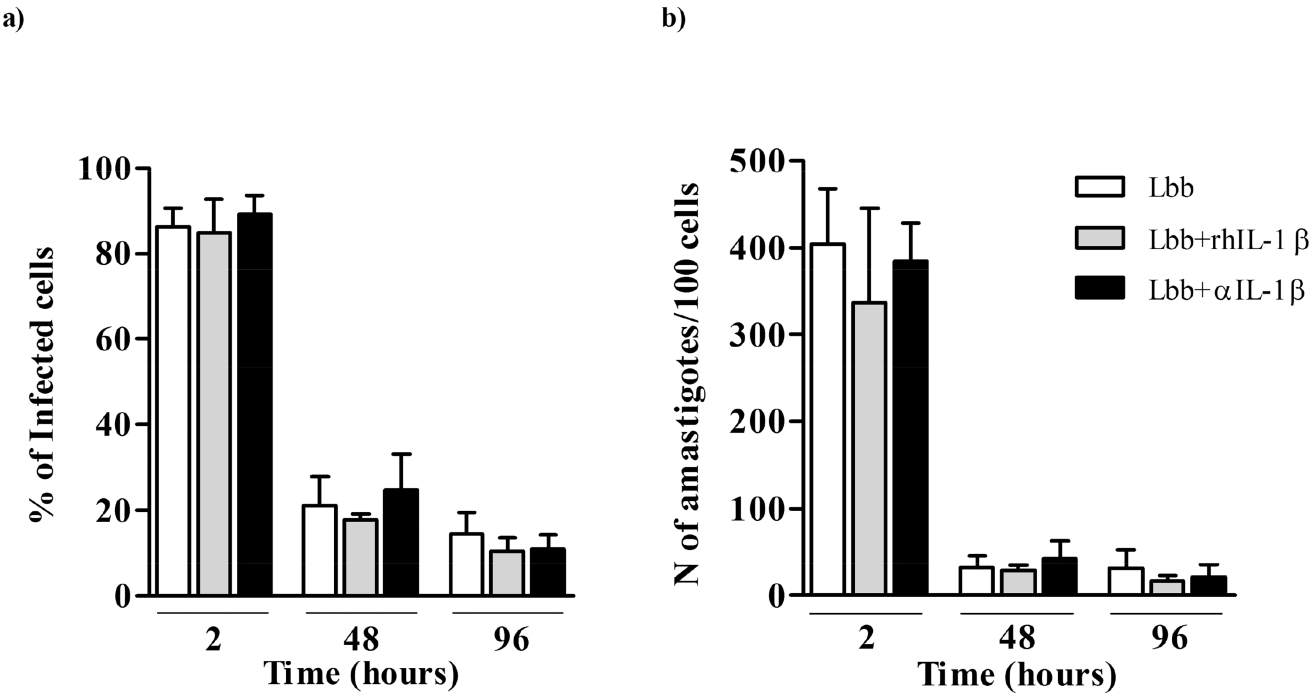

Figure 6. Phagocytosis and killing of *Leishmania* does not depend on presence of IL-1β

Monocytes-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 (5μg/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 ± SD.