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Title Page

Glyburide, a NLRP3 inhibitor, decreases inflammatory response and is a candidate to reduce pathology in *Leishmania braziliensis* infection

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LETTER TO THE EDITOR

Cutaneous leishmaniasis (CL) caused by *Leishmania braziliensis* is characterized by an exaggerated inflammatory response that leads to parasite control, but is also the cause of tissue damage and ulcer formation (Carvalho et al., 2012). The NLRP3 Inflammasome is an intracellular protein complex which is activated by pathogen associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs), promoting activation of caspase 1 and release of active IL-1β (Latz et al., 2013). In C57BL/6 mice infected with a nonhealing *L. major* strain, NLRP3 inflammasome and IL-1β were associated with severe pathology (Charmoy et al., 2016) and IL-1β cause severe disease in BALB/C mice infected with *L. major* (Voronov et al., 2010). Recently, we showed that CD8 T cells induce immunopathology by NLRP3 inflammasome activation and IL-1β production in *L. braziliensis* infected mice, and inhibitors of NLRP3 reduces pathology (Novais et al., 2017).

Glyburide is a FDA-approved ATP-sensitive K^+channels inhibitor used for the treatment of type 2 diabetes. Recently, it was shown that glyburide has anti-inflammatory effects mainly by inhibition of NLRP3 inflammasome and decreasing IL-1β release. (Lamkanfi et al., 2009). Furthermore, anti-*Leishmania* activity by glyburide was previously documented (Ponte-Sucre et al., 1997; Serrano-Martín et al., 2006). Additionally, glyburide may act in synergy with pentavalent antimony (Sb^v) promoting *L. major* killing (Padrón-nieves et al., 2009).

Here, we evaluated the ability of glyburide to reduce the systemic and local inflammatory response of CL patients and it's leishmanicidal effect against intracellular amastigotes of *L. braziliensis*.

This study was conducted in an area of *L. braziliensis* transmission and participants include 11 patients with CL. This study was approved by the Ethical Committee of the

Federal University of Bahia Medical School and written informed consent was obtained from all participants. A detailed description of the methods is presented in the Supplementary Materials. Peripheral blood mononuclear cells (PBMC) were cultured with soluble *Leishmania* antigens (SLA), in the presence or absence of glyburide. Unstimulated cells produced low or undetectable levels of cytokines. The levels of *Leishmania*-specific IL-1β significantly decreased after addition of glyburide in a dose dependent manner (Figure 1A). Overall, the median of IL-1β suppression was 72%, 86% and 97% at the concentrations of 50, 100 and 200μM, respectively. IL-17 and TNF levels also decreased in the presence of the drug (Figure 1B and 1C). The percentage of suppression of IL-17 and TNF with 200μM of glyburide was 95% and 69%, respectively. No change in IFN-γ, IL-6 and IL-10 levels were observed (Figure 1D-F).

Next, we evaluated the ability of glyburide to down-modulate the inflammatory response in *L. braziliensis* lesions. Addition of glyburide decreased the levels of IL-1β (Figure 2A), IL-17 (Figure 2B), and TNF (Figure 2C) by 95%, 65% and 83%, respectively. There was no change in the levels of IFN-γ, IL-6 and IL-10. These results indicated that glyburide was able to reduce both systemic and tissue inflammatory response in CL patients without toxic effects to host cells (Supplementary Figure S1).

As an exaggerated inflammatory response is associated with pathology in *L. braziliensis* infection, we performed a correlation between cytokine levels in biopsy cultures with lesion size and illness duration. Despite the small sample size, we found a positive correlation between IL-1β levels with lesion size ($r=0.6$, $p=0.04$) and with illness duration (Supplementary Figure S2 A and B).

Next, we evaluated the anti-amastigote activity of glyburide in *L. braziliensis* infected macrophages. Treatment with glyburide did not modify the infection rates, nor impair

the ability of Amphotericin B (AMB) to kill *L. braziliensis* in infected macrophages (Supplementary Figure S3 A and B).

Macrophage activation by IFN-γ and TNF is the main defense mechanism against *Leishmania* parasites. However, an exaggerated inflammatory response may lead to tissue damage and consequently the development of ulcerated lesions. NLRP3 inflammasome is involved in activation of caspase 1, which is important for maturation and secretion of IL-1β. Here, we found a modulatory activity of glyburide in *Leishmania*-specific IL-1β levels in a dose dependent manner. In PBMC IL-1β only occurred in SLA stimulated cultures but glyburide act mainly in monocytes as IFN-γ production was not affected. Actually, IL-1β production in CL seems dependent on NLRP3 activation. This result is in concordance with our previous finding in mice infected with *L. braziliensis* treated with glyburide or in mice deficient for NLRP3 (Novais et al., 2017; Santos et al., 2018). In addition, NLRP3 inflammasome and IL-1β have been associated with disease severity in leishmaniasis both in mice and humans (Novais et al., 2017; Fernandez-Figueroa et al., 2012; Santos et al., 2018). Our documentation of a positive correlation between IL-1β levels and lesion size although with a small number of patients evaluated, argues in favor that IL-1β overproduction promotes pathology in human CL caused by *L. braziliensis*. Moreover, we found that high dose of glyburide induced a significative reduction of IL-17 and TNF levels, inflammatory cytokines that are also associated with pathology in *Leishmania* infection (Gonzalez-Lombana et al., 2013; Carvalho et al., 2013).

Here, we didn't find any change in infection rates of *L. braziliensis* in macrophages after treatment with glyburide. This is agreement with our previous observation that treatment with glyburide did not affect parasite load in *L. braziliensis* infected mice (Novais et al., 2017). In contrast, deficiency in NLRP3 inflammasome was associated

with a decrease in NO production and impaired parasite control in mice infected with *Leishmania amazonensis* (Lima-Junior et al., 2013). As NO seems to be less important to *L. braziliensis* killing in humans (Carneiro et al., 2016), it is likely that NLRP3 inflammasome does not play an important role in parasite control in CL patients due *L. braziliensis*.

The standard drug for CL treatment is Sb^V, a highly toxic drug with growing therapeutic failure rates. Since the inflammatory response is a major cause of pathology in CL, a combination therapy composed by an anti-*Leishmania* drug plus an anti-inflammatory agent should be considered. Here, we showed that in vitro addition of glyburide, decreases the inflammatory response without decreasing IFN-γ production, suggesting that this drug may reduce pathology without affecting the main mechanism of parasite control. Therefore, we encourage clinical trials using glyburide as adjuvant therapy to treat CL.

DATA AVAILABILITY STATEMENT

Datasets related to this article can be found at https://figshare.com/s/2646c9c1d837bfd8d790, hosted at Figshare.

CONFLICT OF INTEREST.

The authors state no conflict of interest.

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CREDIT STATEMENT

Author Contributions

Conceptualization: AMC; LPC; PS; EMC.

Data curation: AMC.

Formal analysis: AMC.

Funding acquisition: LPC; PS; EMC.

Investigation: AMC; FON; CSP; CIdO; PRLM;LPC;EMC.

Methodology: AMC; LPC; PS; EMC.

Project administration: EMC.

Resources: LPC; CIdO; PRLM; EMC.

Supervision: EMC.

Validation: AMC; FON; CSP.

Visualization: AMC; LPC; PS; EMC.

Writing – original draft: AMC.

Writing – review & editing: LPC; CIdO; PRLM; PS; EMC.

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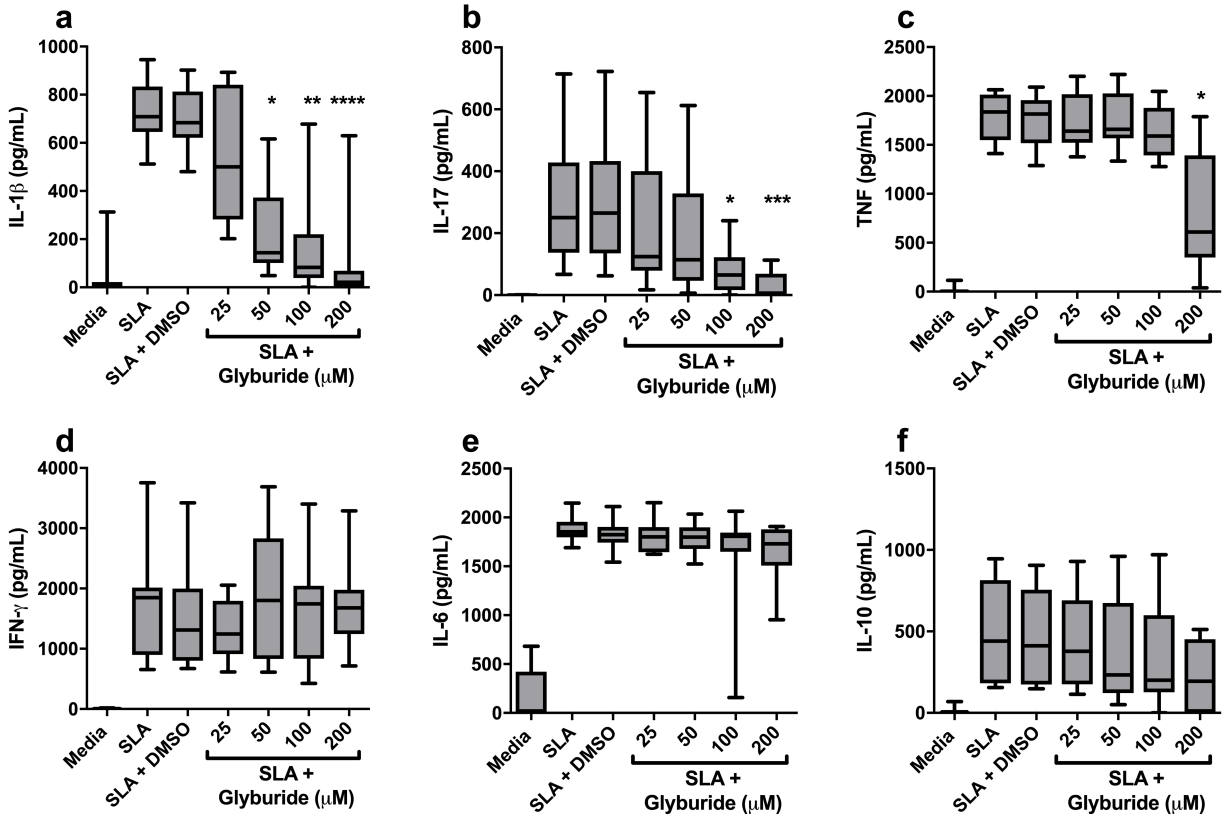
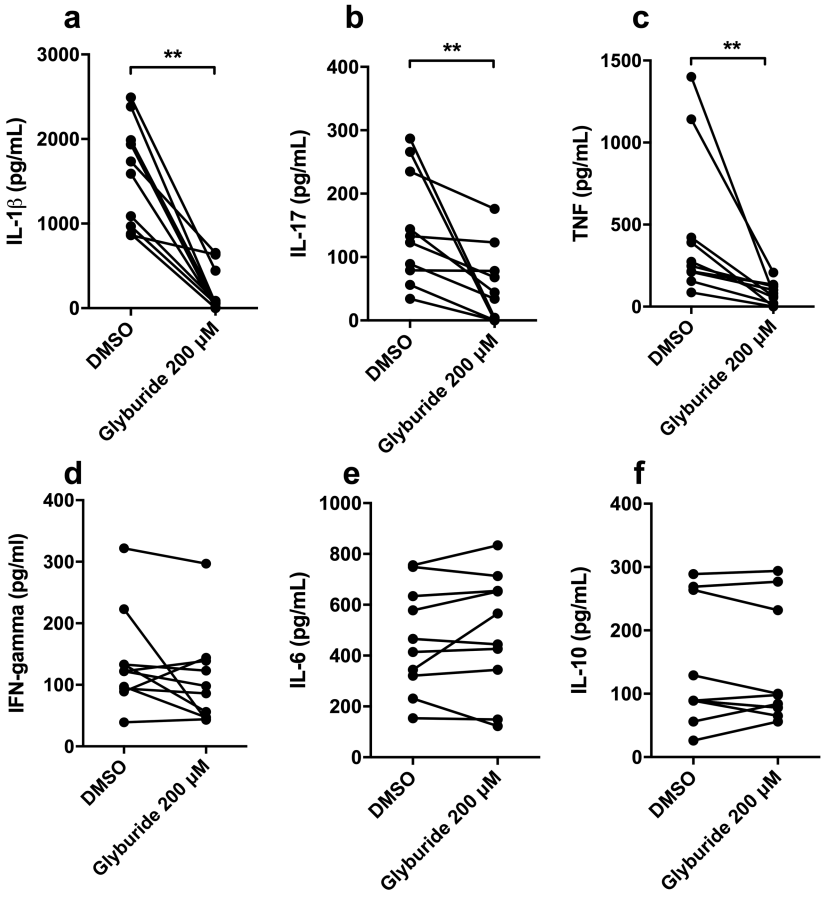
FIGURE LEGENDS

Figure 1. Glyburide down modulate SLA-induced cytokine production in PBMC from CL patients. Peripheral blood mononuclear cells (PBMC) from 11 CL patients were cultured in presence of soluble *Leishmania* antigen (SLA) (5µg) plus glyburide in different concentrations or plus drug diluent (DMSO) for 72 hours. Cytokine levels in culture supernatants were measured by ELISA. Levels of IL-1β (A), IL-17 (B), TNF (C), IFN-γ (D), IL-6 (E) and IL-10 (F). Central lines represent median values. *P < 0.05; **P < 0.01; ****P < 0.0001.

Figure 2. Glyburide down modulate pro-inflammatory cytokine release from lesions biopsies of CL patients. *L. braziliensis* lesions skin biopsies from 11 CL patients were cut in half, and one half was cultured in media plus DMSO and the other half with media plus glyburide (200µM) for 48 hours. Cytokine levels in biopsies supernatants were measured by ELISA. Levels of IL-1β (A), IL-17 (B), TNF (C), IFN-γ (D), IL-6 (E) and IL-10 (F). **P < 0.01.





SUPPLEMENTAL MATERIAL

Area of study and selection of individuals

This study was conducted in Corte de Pedra, Bahia, Brazil, a well-known area of *L. braziliensis* transmission. Participants include 11 patients with CL who had a typical ulcerated cutaneous lesion, and documentation of DNA for *L. braziliensis* by PCR. All the immunologic studies were performed prior to therapy. All individuals were treated with meglumine antimoniate (20 mg/kg/day) for 20 days.

Cell culture and determination of cytokines

PBMC were isolated from heparin-treated venous blood by ficoll-hypaque gradient centrifugation. After washing three times in 0.9% NaCl solution, cells were re-suspended in RPMI 1640 culture medium (GIBCO BRL, Grand Island, NY) supplemented with 10% human AB serum, 100 IU/ ml of penicillin and 100 µg/ml of streptomycin. Cells were adjusted to 3×10^6 cells/ml, put in 24-well plates and cultured with SLA (5 µg/ml), SLA plus glyburide (SIGMA) (25 – 200 µM) or drug vehicle (DMSO). After incubation for 72 hours at 37°C and 5% CO_2, supernatants were collected and stored at –20°C. Biopsies from *Leishmania* lesions were cut into two pieces and weighed. Half of the biopsies were cultured in media containing drug vehicle (DMSO) and the other half were cultured with 200µM of glyburide (SIGMA) for 48 hours at 37°C and 5% CO_2, supernatants were collected and stored at –20°C. The cytokine levels were measured by ELISA (R&D Systems, Minneapolis, MN) sandwich method and the results expressed as pg/ml.

Trypan blue exclusion assay

PBMC (1×10^6) or macrophages (3×10^5) were cultured in a 24-well plate in the presence of different concentrations of glyburide (SIGMA) or Sodium Azide 2% as

positive control. After 48 hours of treatment, 0.03% trypan blue was added, and the number of viable cells was estimated by counting 100 cells in triplicate.

Anti-amastigote activity of glyburide

PBMCs (3×10^6) were plated onto 4 well Lab-Tek chamber slides (Thermo Scientific) and incubated for 2 hours at 37°C and 5% CO_2. Nonadherent cells were removed; adherent cells were cultured for another 6 days. Macrophages were infected with stationary phase of *L. braziliensis* (MHOM/BR/00/2000) (5 parasites to 1 macrophage) for 2 hours at 37°C and 5% CO_2. Noninternalized parasites were removed, and infected macrophages were further cultured alone or in presence of glyburide (100 and 200 µM), drug vehicle (DMSO), and AMB (0.25 µg) for 2 and 48 hours. Slides were washed, stained with hematoxylin-eosin, and analyzed with light microscopy for determination of the percentage of infected cells and the number of amastigotes per 100 cells

Statistical analysis

Comparisons between 2 groups were performed by Mann–Whitney test and among 3 or more groups by Kruskal–Wallis test followed by Dunn multiple comparison tests. Correlation between IL-1β levels and lesion size or illness duration was evaluated using non-parametric Spearman test. Statistical analyses were conducted using Prism (V. 5.0) (GraphPad Software) and differences were considered significant when $P < .05$.

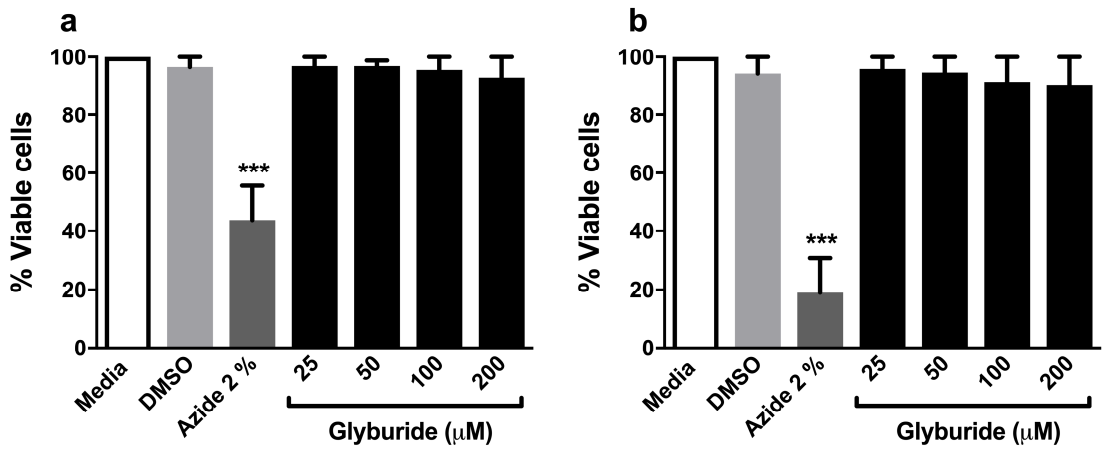


Figure S1. Cytotoxicity of glyburide against human PBMC and macrophages. PBMCs (1×10^6) or macrophages (3×10^5) from 5 healthy subjects were cultured in the presence or absence of glyburide or diluent (DMSO) for 48 hours at 37°C in 5% CO_2. Sodium azide was used as a positive control. Viability was measured using a trypan blue exclusion assay. The results represent the median and range. ***P < 0.001 compared to the control (Medium).

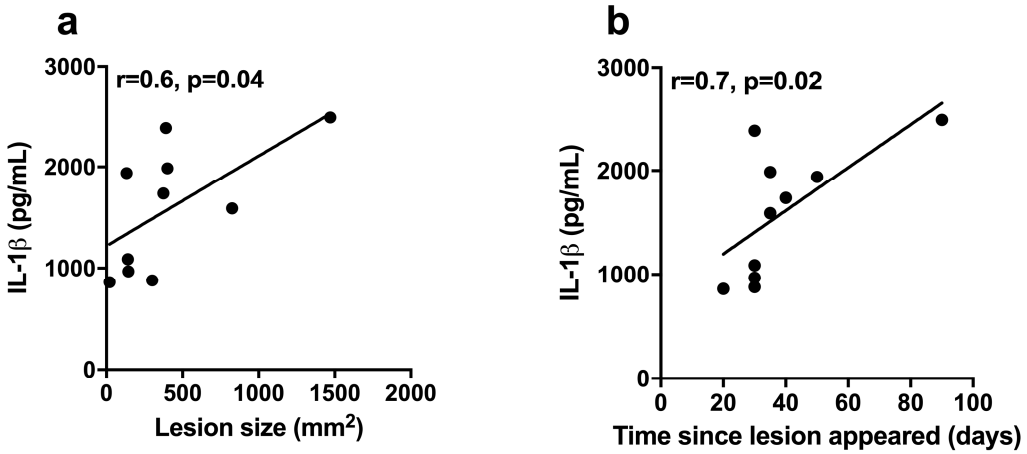


Figure S2. Correlation between IL-1β levels in lesions biopsies and lesion size or time since lesion appearance. *L. braziliensis* lesions skin biopsies from 11 CL patients were cut in half, and one half was cultured in media plus DMSO and the other half with media plus glyburide for 48 hours. IL-1β levels in culture supernatants were measured by ELISA. Correlation between IL-1β levels in and lesion size (A). Correlation between IL-1β levels and time since lesion appearance (B).

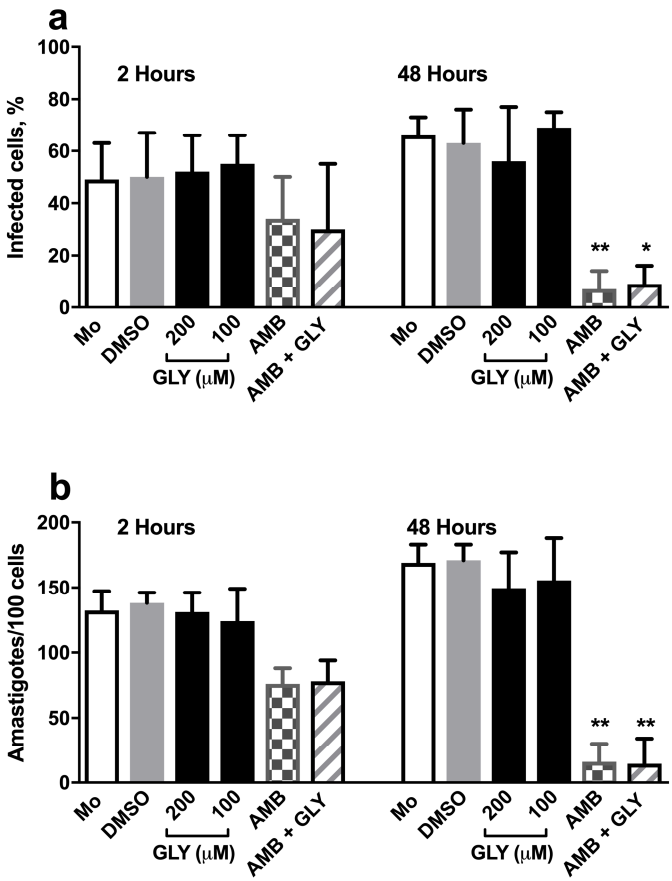


Figure S3. Leishmanicidal effect of glyburide on amastigote-infected macrophages. Macrophages from 7 CL patients were infected with *L. braziliensis* promastigotes at a ratio of 5 parasites to 1 macrophage for 2 and 48 hours in the presence or absence of glyburide (GLY) (100-200 μM), amphotericin B (AMB) (0.25 μg/ml) or drug diluent (DMSO). Glass coverslips were stained with hematoxylin-eosin and the percentage of infected macrophages (A) and the number of amastigotes per 100 macrophages (B) were determined by light microscopy. The results represent the median and range. *P < 0.05; **P < 0.01 compared to untreated control.