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High glucose heightens vulnerability to *Leishmania braziliensis* infection in human macrophages by hampering the production of reactive oxygen species through TLR2 and TLR4

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ABSTRACT

Diabetes increases susceptibility to infections, including *Leishmania braziliensis* (*Lb*). Our group previously demonstrated that diabetic patients with cutaneous leishmaniasis (CL) take longer to heal lesions compared to non-diabetics. Since macrophages play a critical role in CL pathogenesis, we investigated how high glucose levels impact their response during *Lb* infection. Macrophages cultured in high glucose conditions showed increased parasite load than those in normal glucose conditions. The production of inflammatory mediators was similar between glucose conditions, but basal reactive oxygen species (ROS) production was elevated under high glucose conditions and remained unchanged after *Lb* infection, indicating glucose-induced oxidative stress does not control the parasite. In contrast, macrophages in normal glucose conditions, exhibited increased ROS production only after infection. Additionally, high glucose reduced TLR2 and TLR4 expression, which was also observed after *Lb* infection. TLR2/4 inhibition increased *Lb* infection in normal glucose conditions, mediated by TLR-dependent ROS production. However, this mechanism was absent under high glucose conditions, where elevated basal ROS production appeared TLR-independent. Biopsies from diabetic CL patients corroborated these findings, showing decreased TLR2 and TLR4 expression compared to non-diabetics. These findings suggest that high glucose levels induce oxidative stress and reduces TLR expression, impairing macrophage functions and rendering them less effective at controlling Lb infection.

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KEYWORDS Leishmaniasis; diabetes; macrophages; TLRs; ROS; glucose

Introduction

Leishmaniasis is a complex disease that represents a significant public health concern in more than 80 countries, with an incidence of 19.7 cases per 10,000 inhabitants in Latin America, the majority occurring in Brazil [1]. The etiological agents of the disease are parasites of the *Leishmania* genus, with *L. braziliensis* (*Lb*) being commonly associated with Cutaneous Leishmaniasis (CL).

The immune response during CL is mediated by CD4+ T cell activation through dendritic cell presentation of *Lb* antigens, which stimulate differentiation towards a Th1 profile [2]. These cells produce IFN-γ, which increases the response against *Lb* through innate immunity by activating macrophages to produce reactive oxygen species (ROS) nitric oxide [3,4], and cytokines, such as IL-10, IL-6 and TNF-α.

The skin represents the first barrier between the host and the environment. However, in diabetes mellitus (DM), structural and functional alterations of the skin are common, leading to heightened susceptibility and severity of various dermatological conditions. Approximately 30% of individuals with DM eventually develop skin disorders, predominantly due to infections [5]. This susceptibility has been associated with the systemic effects of DM, which compromises the immune response and impair the skin's barrier function.

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DM is a metabolic syndrome characterized by increased blood glucose levels, either by the absence of insulin production, characteristic of type 1 diabetes, or by decreased production or tissue resistance to insulin, associated with type 2 [6]. It is estimated that 422 million people worldwide have DM, and about 1.6 million deaths are associated with the disease each year. Both types of DM are associated with secondary complications, such as vascular changes, retinopathies, nephropathy, heart disease, and diabetic neuropathy, as well as increased susceptibility to infections, especially of the skin [7,8]. Despite the chronic low-grade inflammation observed in DM, individuals with DM are more susceptible to infectious diseases. However, the mechanism that leads to this increased susceptibility is still poorly understood [8,9].

A study with hospitalized patients showed that for every 1 mmol/l increase in blood glucose, the risk of developing pneumonia, urinary tract infections, and skin infections increases by 6–10% [9]. In 2015, Filgueiras and collaborators [10] demonstrated a strong association between exacerbated inflammation and greater susceptibility to sepsis in a murine model of DM. More recently, our group revealed that individuals with DM are more prone to *Lb* infection despite increased circulating levels of inflammatory mediators [11]. In this previous study, we found that reactive oxygen species (ROS) production is compromised in macrophages obtained from CL patients with DM compared to non-DM patients, leading to an increased parasite load after *Lb* infection *in vitro*. The primary mechanism by which macrophages control *Lb* infection is through ROS generation [12], and it is well established that its production can be triggered by interactions with membrane receptors, including Toll-like receptors (TLRs). That interaction promotes NF-kB activation, leading to enhanced inflammation, oxidative stress and the subsequent killing of microorganisms [4].

Nevertheless, how hyperglycaemia regulates ROS production and parasite control in human cells remains unknown. In the present study, we cultured monocyte-derived human macrophages under different glycemia conditions before the infection with *Lb* to identify mechanisms involved in ROS and cytokine production and parasite control. We found that increased glucose levels induce a basal production of ROS and a reduction in TLR2 expression, rendering macrophages more prone to *Lb* infection. Additionally, TLR4 expression was reduced in biopsies from patients with LC and diabetes, reinforcing previous *in vitro* findings.

Methods

Ethics statements

The Institutional Review Board for Ethics in Human Research at the Gonçalo Moniz Institute (Oswaldo Cruz Foundation IGM-FIOCRUZ, Salvador, Bahia-Brazil) approved this study (protocol number: CAAE 34954814.1.0000.0040 and CAAE: 38573414.1.0000.0040). Sample collection was performed in accordance with ethical regulations, in the endemic areas for leishmaniasis (Corte de Pedra and Jiquiriçá, Bahia, Brazil).

Study population selection

Eleven subjects with confirmed CL and 10 with both CL and diabetes, all with their respective diagnosis confirmed, were selected from the Municipal Health Clinic in Corte de Pedra and Jiquiriçá. Groups were paired trough age and sex. CL diagnosis was performed from clinical and histopathological analyses, such as isolation of parasites or Polymerase Chain Reaction (PCR) for parasite DNA detection and DTH to leishmania antigen (Montenegro skin test) [13]. To confirm diabetes diagnosis fasting glucose levels were used, and individuals with levels ≥126 mg/dL were considered with diabetes. Exclusion criteria were pregnant women, children under 15 years old and negative PCR for parasite DNA. All samples were collected after CL diagnosis and before treatment. Following Brazilian Ministry of Health, treatment of CL was performed using Glucantime, and patients with diabetes were treated with Metformin. Clinical and epidemiological data were obtained from included individuals (see Table 1).

Sample collection

Blood samples were drawn by venipuncture using vacuum tubes with Heparin. Cell obtainment and culture was performed as previously described [14]. Briefly, collected blood was diluted 1:1 in saline solution to obtain peripheral blood mononuclear cells (PBMCs) using HISTOPAQUE® 1077 gradient reagent (Sigma Aldrich, St Louis, MO). PBMC layer was obtained by centrifugation for 30 min at 400 g. Skin biopsies were obtained from CL subjects from the endemic area with and without diabetes, using a 4 mm punch, and preserved in *RNALater* (Invitrogen, Waltham, MA).

Culture of human macrophages

PBMCs from healthy donors were plated (2×10^6) on 24-well tissue culture plates (TPP, Switzerland) with

Table 1. Clinical data from patients with LCL and LCL+DM.

Group	LCL ($n = 11$)	LCL + DM ($n = 10$)	p value
Gender	Male (77.8%) Female (22.2%)	Male (42.9%) Female (57.1%)	0.3024
Age	38 ± 24.1	45 ± 15.3	0.4913
Lesion size (mm^2)	17.5 (10–26.5)	60 (17.5–640)	**0.0488**
Time to healing (days)	39 (30–143.3)	74.5 (23.25–146.5)	0.9744

Data shown as mean ± SD and median (IQR).
Bold values indicate statistical significance (p-values <0.05).

13 mm round glass coverslips (Perfecta) and incubated with RPMI medium (Thermo Fisher Scientific, Waltham, MA) at 37°C under 5% CO_2 for 30 min. Adherent cells were cultured in RPMI medium, with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA), 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/mL streptomycin, 50 ng/mL M-CSF (Peprotech) and glucose and mannitol (Sigma Aldrich, St Louis, MO) in different concentrations (90, 150 and 300 mg/dL) for 7 days to obtain monocyte-derived macrophages. The highest glucose concentration was established based on the average blood glucose levels of patients with Cutaneous Leishmaniasis from the endemic area of Corte de Pedra, Bahia, Brazil. Cell viability was evaluated in different glucose concentrations (90, 150, 300, 450, 600, 750 and 900 mg/dL) by Alamar blue colorimetric-based assay, according to the manufacturer's instructions.

Culture of Leishmania braziliensis *and macrophage infection*

L. braziliensis (MHOM/BR/01/BA788) promastigotes were cultured in Schneider's Insect medium (Sigma Aldrich, St Louis, MO) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin at 24°C. Cultured macrophage were infected with stationary-phase *L. braziliensis* for 4 h at a parasite/cell ratio of 10:1. Supernatant were harvested, cells were fixed and stained with Quick Panoptic (LB Laborclin, Paraná, Brazil). Blinded counts of 100 random cells were performed by microscopy under an objective (Nikon) at 100x to determine infection rate. Parasite viability was assessed by adding Schneider's medium to the human macrophage culture and counting live promastigotes after 72 h.

Quantification of inflammatory mediators

Quantification of TNF-α (Invitrogen, San Diego, CA) and IL-6 (eBioscience, San Diego, CA) cytokines and LTB_4 and PGE_2 (Cayman Chemical, Ann Arbor, MI) lipid mediators were performed using enzyme-linked immunosorbent assays (ELISA), according to the manufacturer's instructions.

Reactive oxygen species production

ROS production was assessed in human monocyte-derived macrophages, both with and without *Lb* infection, under normal and high glucose conditions. To confirm ROS-dependent *Lb* killing, cells cultured at 90 mg/dL glucose were treated with 5 mM/mL N-Acetyl-L-cysteine (NAC, Sigma Aldrich), an antioxidant that neutralizes ROS, prior to infection with *Lb* for 4 hours. Additionally, cells were pre-incubated with TLR2 and TLR4 antagonists (α-TLR2 and α-TLR4, InvivoGen, San Diego, CA) at 100 µg/mL for 1 h to validate the involvement of these receptors in ROS generation, followed by *Lb* infection for 4 hours.

Cells were incubated with 5 µM of CellROX™ Green Reagent (Thermo Fisher Scientific, IL, USA) for 30 min at 37°C and 5% CO_2, then washed with 1x PBS (Gibco), fixed and stained with DAPI and ProLong Gold antifade reagent (Thermo Fisher Scientific, IL, USA). Images were captured by fluorescence microscopy, with 485/520 excitation. Corrected total cell fluorescence (CTCF) was calculated based on an average of 24 cells per subject (eight randomly selected cells per field), and 5 adjacent fields to determine the background mean, using ImageJ 1.52a software (National Institutes of Health, USA).

Gene expression analysis

The relative expression of TLR2 and TLR4 (IDT, Coralville, IA) was evaluated in human monocyte-derived macrophages and skin biopsies from CL lesions. Total RNA was extracted using the miRNeasy Mini Kit (QIAGEN, Hilden, Germany), and cDNA was synthesized with the SuperScript® III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was then performed using SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA). Relative gene expression is presented as fold changes between the control group (90 mg/dL glucose for macrophages or non-diabetic biopsies) and high-glucose groups (150 and 300 mg/dL for macrophages or diabetic biopsies), analyzed using the 2ΔΔCT method. β-Actin (ACTB, assay ID Hs.PT.39a.22214847) served as the housekeeping gene, which is widely used due to its abundance and stability in skin wound models, and to synthesizing β-Actin, a protein present in the cytoskeleton structure [15].

Flow cytometry

Protein expression of TLR2 and TLR4 in human monocyte-derived macrophages was assessed by flow cytometry, both before and after infection. Cells were stained with anti-CD282 (TLR2, BD Biosciences), anti-CD284 (TLR4, eBioscience, San Diego, CA), and anti-CD14 (Invitrogen, Waltham, MA) antibodies. The antibody panel was prepared in PBS containing 1% BSA and incubated for 30 min at room temperature. Data acquisition was performed using a BD FACS Canto II flow cytometer (BD Biosciences), with all compensation and gating analyses carried out using FlowJo version 9.5.3 (TreeStar, Ashland, OR).

Statistical analysis

Data are presented as mean ± standard deviation (SD) or median with interquartile range (IQR) for numerical variables, and as proportions (%) for categorical variables. For

normally distributed variables, comparisons between two groups were made using Student's *t*-test. For non-normally distributed variables, the Mann–Whitney test was used for two groups, and the Kruskal–Wallis test with Dunn's post-test for comparisons involving three or more groups. Correlations were assessed using Spearman's test. For paired variables with normal distribution, Friedman's test was applied. All statistical analyses were performed using Prism 8 software (GraphPad, USA). For multiple comparisons, differences were considered statistically significant when $p < 0.05$.

Results

Human macrophages exhibit heightened susceptibility to infection by L. braziliensis *under high glucose conditions*

To evaluate the effects of glucose on the immune response of human monocyte-derived macrophages to *Lb* infection, cells were exposed to euglycemic conditions (90 mg/dL) or two hyperglycemic conditions (150 and 300 mg/dL) prior to infection. The frequency of infected cells significantly increased at higher glucose concentrations, particularly at 300 mg/dL, compared to macrophages cultured at lower glucose levels (Figure 1(A and B)). In terms of parasite load, macrophages cultured at 300 mg/dL harboured a greater number of amastigotes per cell (Figure 1(C)) than those under normoglycemic conditions. Additionally, post-infection analyses showed that macrophages exposed to high glucose levels were less effective at eliminating parasites (Figure 1(D)). These findings suggest that monocyte-derived macrophages cultured under hyperglycemic conditions are more susceptible to *Lb* infection and exhibit a reduced capacity to control parasite proliferation.

L. braziliensis *elicits the production of inflammatory mediators by macrophages, whereas high glucose alone does not*

To assess the inflammatory response triggered by varying glucose concentrations and/or *Lb* infection, cytokines and lipid mediators were measured in the supernatant of cultured macrophages. After 4 h, high glucose alone did not stimulate the release of any mediator. However, infection with *Lb* led to a significant induction of LTB_4 (Figure 2(A)), TNF-α (Figure 2(C)) and IL-6 (Figure 2(D)). Interestingly, PGE_2 levels were unaffected by both glucose concentration and *Lb* infection (Figure 2(B)). Regardless of glucose levels, these findings reveal a distinct inflammatory profile in macrophages infected with *Lb*.

High glucose levels induce ROS production, which remains unchanged following L. braziliensis *infection*

To explore the effects of glucose levels on ROS production, we used fluorescence dyes to measure ROS

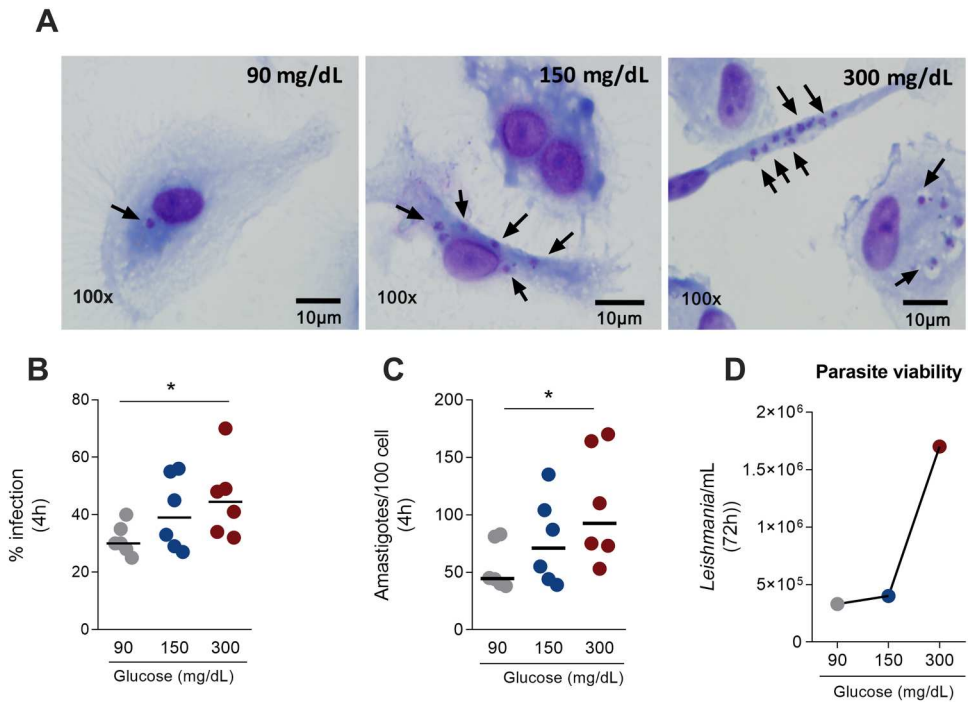

Figure 1. Increased susceptibility of human macrophages to *L. braziliensis* infection under high glucose. (A) Human macrophages from healthy donors were cultured in different glucose concentrations (90, 150, 300 mg/dL) and infected with *L. braziliensis* for 4 h. (B) Infection rate of macrophages under different glucose conditions. (C) The total number of amastigotes per 100 cells cultured in different glucose concentrations and infected by *L. braziliensis*. (D) Parasite viability after 72 h of infection. (A-C, $n = 6$; D, $n = 13$). (B, C = ANOVA Test). Arrows: amastigotes. *$p < 0.05$.

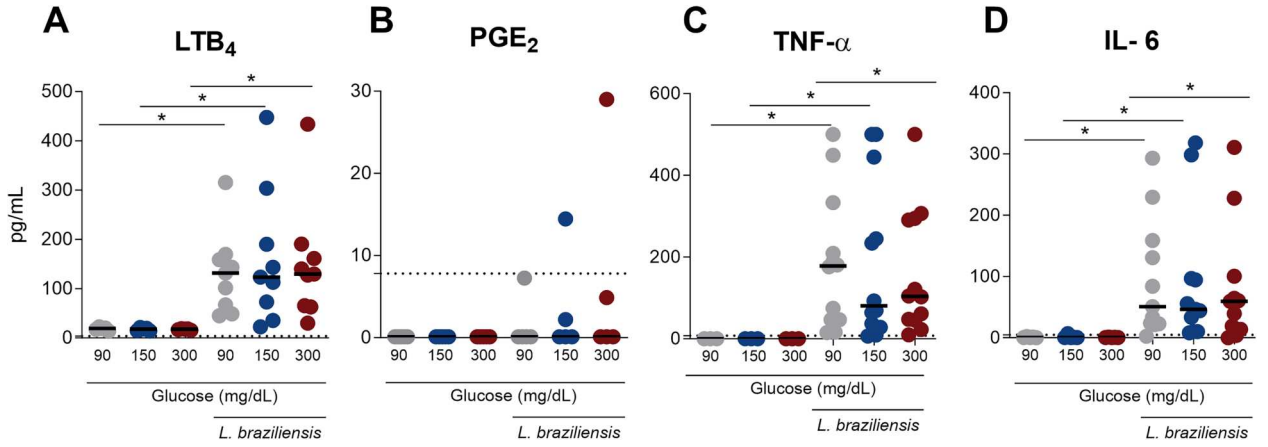

Figure 2. High glucose does not influence the production of inflammatory mediators in human macrophages infected with *L. braziliensis*. Levels of (A) LTB_4, (B) PGE_2, (C) IL-6 and (D) TNF-α in the supernatant of human macrophages cultured at different glucose concentrations and infected with *L. braziliensis* for 4, measured by ELISA. (A, glucose $n = 5$; glucose + infection $n = 9$); (B, glucose $n = 5$; glucose + infection $n = 5$); (C, glucose $n = 5$; glucose + infection $= 11$); (D, glucose $n = 3$; glucose + infection $n = 12$). (A–D = Mann–Whitney test). *$p < 0.05$.

in macrophages cultured under varying glucose concentrations, both infected and uninfected. Significantly higher ROS levels were observed in cells cultured at 150 and 300 mg/dL compared to those at 90 mg/dL, indicating that high glucose induces oxidative stress independent of infection (Figure 3(A, B)). However, following *Lb* infection, ROS production significantly increased at 90 mg/dL, while it remained unaltered under high glucose conditions (Figure 3(A–D)). Additionally, the ratio of ROS production between infected and uninfected cells at 300 mg/dL did not increase as it did at 90 mg/dL (Figure 3(C)), suggesting that the reduced capacity to control parasites in high glucose conditions (Figure 1) may be attributed to the lack of ROS enhancement.

TLR2 and TLR4 expression is decreased in high glucose conditions and following L. braziliensis *infection*

To confirm the role of ROS in parasite elimination, we treated cells with N-acetylcysteine (NAC), a well-known ROS inhibitor. NAC treatment resulted in an increased percentage of infected cells and a higher number of amastigotes (Figure S1A–C), underscoring the critical role of ROS in controlling the infection. Moreover, we found that TLR2 and TLR4 signalling are potent inducers of ROS production under normal glucose conditions. Inhibition of these receptors significantly reduced ROS levels in *Lb*-infected macrophages (Figure S1D and E), confirming that TLR2-

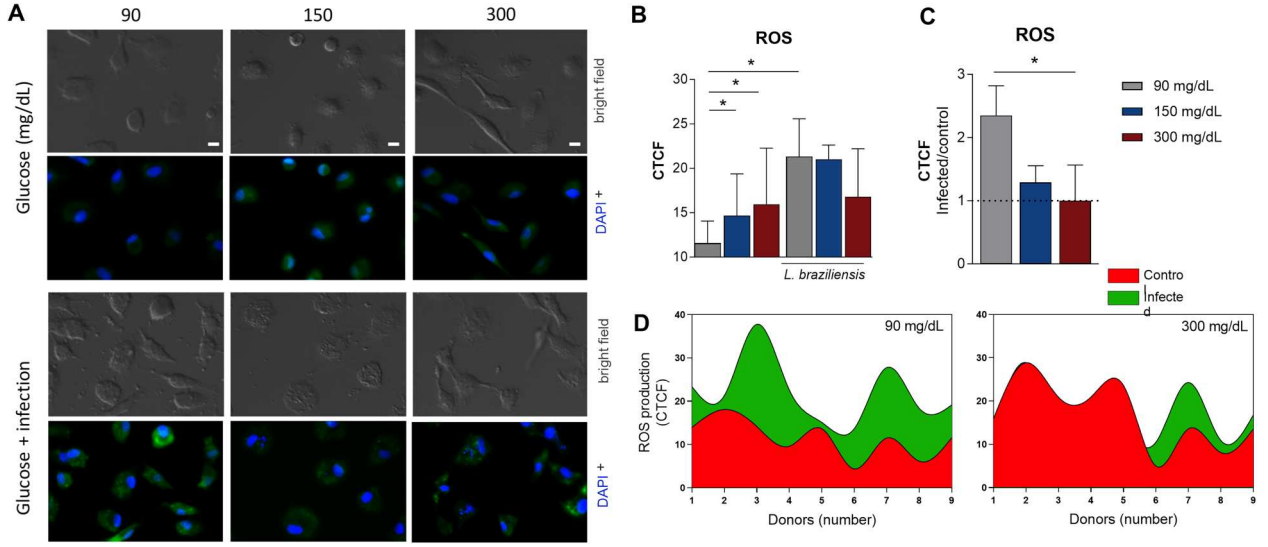

Figure 3. High glucose does not alter ROS production in human macrophages following *L. braziliensis* infection (A) ROS production in human macrophages cultured in different glucose concentrations before and after infection with *L. braziliensis*. (B) Comparative ROS production in human macrophages under different glucose concentrations and *L. braziliensis* infection. (C) ROS production in infected versus control human macrophages. (D) Cubic spline analysis of donor-specific ROS production in macrophages before (red) and after (green) *L. braziliensis* infection. Corrected Total Cell Fluorescence (CTCF) indicates ROS production (green) and cell nuclei (blue) at 40x magnification. Scale bar = 10 µm. (B) Wilcoxon's test. (C) Friedman's test. Data is shown in median with interquartile range (C). *$p < 0.05$.

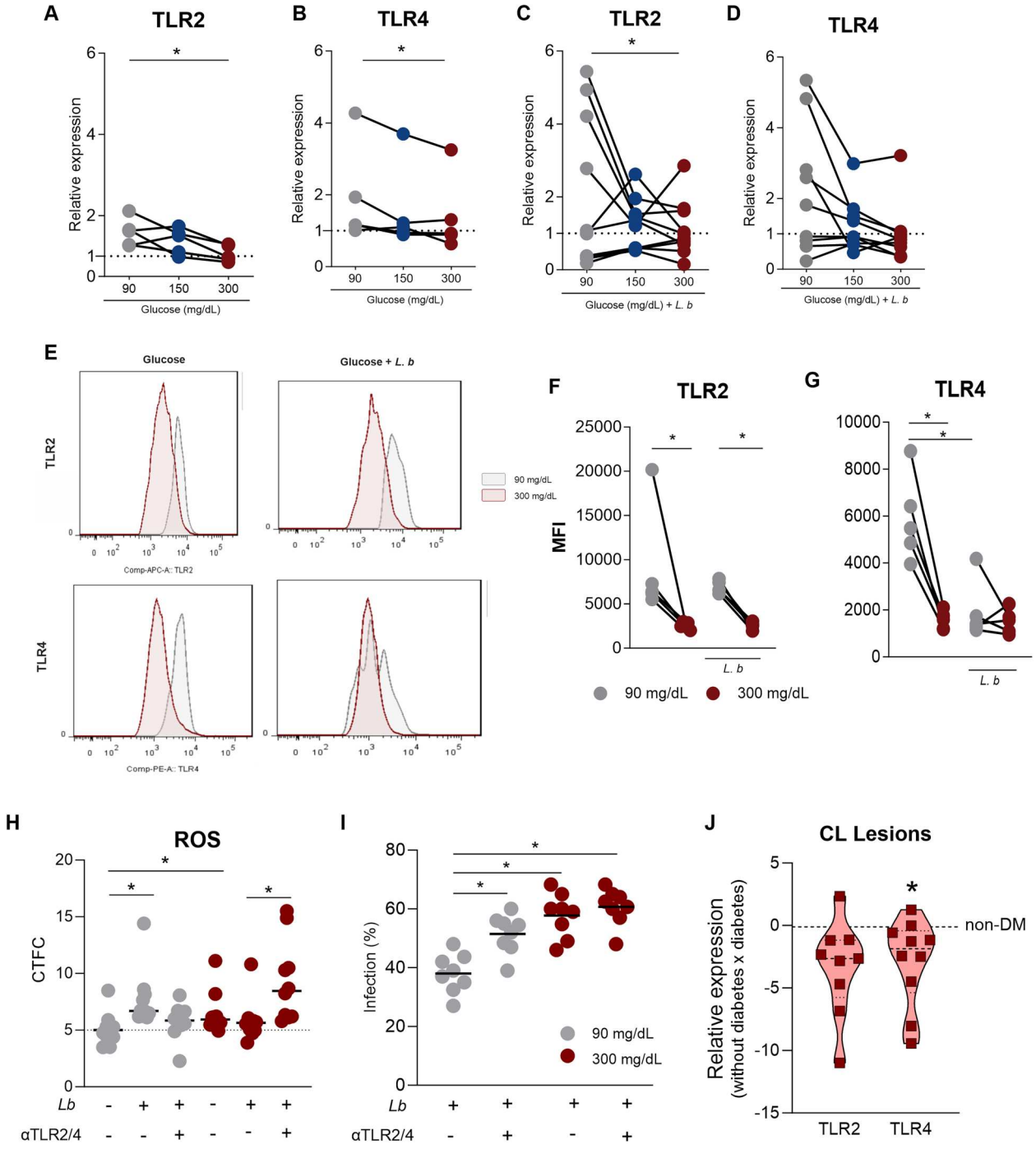

Figure 4. High glucose inhibits TLR2 and TLR4 expression in human macrophages during an *L. braziliensis* infection. Relative expression (RT-qPCR) of TLR2 (A) and TLR4 (B) in human macrophages cultured in different glucose concentrations. Relative TLR2 (C) and TLR4 expression (D) in human macrophages infected with *L. braziliensis* and cultured in different glucose concentrations. (E) Flow cytometry histogram of TLR2 and TLR4 in human macrophages cultured in different glucose concentrations and infected with *L. braziliensis*. Mean Fluorescence Intensity (MFI) of TLR2 (F) and TLR4 (G). (H) ROS production in human macrophages treated or not with α-TLR2/4, before and after infection. Dotted line at the control (90 mg/dL) mean (I) Infection rate of macrophages under different glucose conditions, treated or not with αTLR2/4. (J) Relative expression of TLR2 and TLR4 in CL lesions from diabetic (DM) and non-diabetic (NDM) patients. CTFC: Corrected Total Cell Fluorescence (A–C, H,I) Friedman's test. (F,G) Mann–Whitney test. (J) Student's *t* test *$p < 0.05$.

and TLR4-mediated ROS production is essential for parasite killing.

We next assessed TLR2 and TLR4 expression in macrophages cultured in 90, 150, and 300 mg/dL glucose for 7 days. Both receptors were significantly downregulated in cells cultured at 300 mg/dL compared to those in normoglycemic conditions (Figure 4(A and B)), suggesting that high glucose concentrations negatively modulate TLR2 and TLR4 expression. To rule out the possibility of osmotic imbalance affecting the cells, we evaluated *Lb* infection in macrophages exposed to varying concentrations of mannitol, a metabolically inert sugar. The results showed that osmotic imbalance did not interfere

with *Lb* infection (Figure S2A and B), confirming that the observed effects are glucose-dependent. Furthermore, macrophage viability was maintained even at higher glucose concentrations (Figure S2C).

Even after *Lb* infection, TLR2 gene expression remained reduced in hyperglycemic conditions (300 mg/dL) (Figure 4(C)), while TLR4 showed only a slight decrease under the same conditions (Figure 4(D)). At the protein level, similar results were observed: TLR2 and TLR4 expression was significantly lower in macrophages cultured in 300 mg/dL glucose (Figure 4(E–G)). Following *Lb* infection, TLR2 protein levels were also significantly reduced in high glucose (Figure 4(F)), whereas TLR4 showed no significant change (Figure 4(G)).

To confirm the roles of TLR2 and TLR4 in ROS production and *Lb* elimination, we treated human macrophages cultured in 90 and 300 mg/dL glucose with TLRs antagonists. In normoglycemic conditions, *Lb* infection significantly increased ROS production, which was reduced at baseline with TLR2/4 blockade (Figure 4(H)). This reduction was further evidenced by an increased infection rate following TLR2/4 inhibition at 90 mg/dL glucose (Figure 4(I)). In contrast, macrophages cultured in hyperglycemic conditions exhibited stable ROS levels after infection, regardless of TLR2/4 inhibition. Notably, *Lb* infection combined with TLR2/4 blockade under hyperglycemia led to a paradoxical increase in ROS levels, likely driven by oxidative stress (Figure 4(H)). However, the infection rate remained unchanged at 300 mg/dL glucose, irrespective of TLR inhibition (Figure 4(I)). These findings underscore the critical involvement of TLR2 and TLR4 in ROS production and their contribution to *Lb* elimination. Furthermore, hyperglycemia impairs TLR2/4-mediated responses, diminishing the macrophages' ability to control *Lb* infection.

To translate these findings into a clinical context, we analyzed TLR2 and TLR4 expression in skin biopsies from diabetic and non-diabetic individuals with CL. Our results demonstrated significantly lower TLR4 expression and a trend toward lower TLR2 reduction in diabetic patients compared to non-diabetic CL patients (Figure 4(J)), aligning with the *in vitro* results. These findings emphasize that elevated glucose levels can downregulate key TLR receptors, potentially impairing pathogen recognition and contributing to increased susceptibility and progression of infections, as observed in leishmaniasis.

Discussion

This study demonstrated that human monocyte-derived macrophages are more susceptible to *Leishmania braziliensis (Lb)* infection when cultured in hyperglycemic conditions. This increased susceptibility is likely due to the suppression of TLR2 and TLR4 gene expression at high glucose levels, leading to reduced ROS production and impaired infection control.

Macrophages cultured in elevated glucose concentrations exhibited a higher infection rate and parasite load compared to those cultured in normoglycemic conditions (Figure 1). These findings align with previous results from our group, which showed that macrophages from diabetic patients infected with *Lb* harboured a greater number of internalized amastigotes compared to macrophages from non-diabetic individuals [11]. Moreover, diabetes is known to increase susceptibility to bacterial infections, often linked to impaired wound healing observed in patients with this condition [8,16,17].

Our results showed that *Lb* infection stimulated the production of the cytokines TNF-α, IL-6, and the lipid mediator LTB_4 (Figure 2). Previous studies have demonstrated that while *Lb* infection induces LTB_4 production, it simultaneously reduces the expression of its high-affinity receptor BLT1 as an immune evasion mechanism [18]. TNF-α is well-known for its role in activating the immune response during parasitic infections, particularly through classical monocytes and M1 macrophages [19]. IL-6, on the other hand, has been associated with impairing the immune response against *Lb* and promoting the production of matrix metalloproteinase 1 (MMP1) in cutaneous lesions [20]. In this study, hyperglycemia in macrophage cultures for 4 hours did not stimulate the production of inflammatory mediators. This aligns with previous findings that high glucose levels alone do not induce cytokine production in cell culture [21]. However, earlier work from our group revealed that serum levels of these same cytokines and inflammatory mediators were significantly elevated in macrophage supernatants from diabetic donors [11]. This discrepancy may be due to disease-specific molecular mechanisms that are not replicated in cell cultures from healthy donors.

ROS production is linked to mitochondrial metabolism, oxidase activity, and oxygenases involved in the arachidonic acid pathway, as well as other pathways. Inflammatory receptors such as nucleotide-binding oligomerization domain-like receptors (NLRs) and TLRs play significant roles in this process [22]. ROS can also regulate various signalling pathways, including mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3 K) [23], promoting NF-kB activation and cytokine production [24]. Reactive oxygen and nitrogen species are key components of the leishmanicidal response, primarily produced by activated monocytes and macrophages [3,25,26]. In our study, we demonstrated that high glucose levels induce ROS production but compromise the macrophages' effective response against the parasite (Figures 3 and 4). Oxidative stress plays a critical role in the

pathogenesis of diabetes, being associated with numerous complications and cellular dysfunctions that can lead to cell death [27]. Our results align with existing literature, emphasizing the systemic stress experienced by hyperglycemic macrophages. Furthermore, the ability to eliminate parasites is hindered by cellular dysfunction and exhaustion induced by prolonged exposure to high glucose levels.

In this study, we investigated the pathways through which hyperglycemia may compromise ROS production. The TLR pathway, particularly TLR4, has been linked to ROS production and the activation of NF-kB, leading to the release of proinflammatory cytokines [28]. Our findings indicate that high glucose concentrations inhibit the expression of TLR2 and TLR4 at both the gene and protein levels (Figure 4). Previous research has demonstrated that TLR2 and TLR4 are induced in monocytes under hyperglycemic conditions, resulting in NF-kB activation and subsequent inflammatory cytokine production in human microvascular endothelial cells (HMEC-1) [29,30]. Furthermore, the activation of these receptors has been associated with acute lung injury in the context of systemic inflammatory response syndrome, primarily through ROS production [31].

In the context of *Lb* infection, macrophages cultured under high glucose conditions exhibited reduced TLR2 and TLR4 expression compared to those cultured under normoglycemia conditions. It is well established that, in the immunopathogenesis of leishmaniasis, TLR2 and TLR4 expression is typically upregulated, underscoring their importance in the parasite–host interaction [19]. In our study, elevated glucose levels led to a decrease in the expression of these receptors, which was associated with unaltered ROS production after *Lb* infection. This may be driven by multiple mechanisms, including post-transcriptional or translational changes. Additionally, it is possible that high glucose levels promote the degradation or internalization of these receptors, further impairing the immune response. Hyperglycemia can induce oxidative stress, which has the potential to affect the stability and function of cell surface proteins. For instance, excessive ROS production may activate signalling pathways that lead to the internalization of TLRs, reducing their availability on the cell membrane for interaction with pathogens. Studies have shown that the internalization of pattern recognition receptors, such as TLRs, can be a strategy to attenuate the inflammatory response [32,33] although this may compromise macrophages' ability to detect and respond to infections. Additionally, hyperglycemia may stimulate the expression of proteins involved in receptor degradation, such as ubiquitin ligases, which tag TLRs for proteasomal degradation [34]. This not only reduces TLR expression on the cell surface but also negatively impacts macrophages' ability to mount an effective immune response. Moreover, the presence of inflammatory mediators and cytokines produced in response to hyperglycemia can create an environment that favours TLR downregulation. IL-6, for example, is known to modulate receptor expression and inflammatory responses [35], thus contributing to this downregulation in hyperglycemic contexts.

These combined mechanisms not only reduce TLR expression but also lead to decreased ROS production and immune response, resulting in increased susceptibility to infections, such as those caused by *Lb*. Understanding these processes is crucial for developing therapeutic strategies that can restore macrophage function in diabetic and hyperglycemic individuals, thereby improving immune response and pathogen clearance. Certain drugs, such as statins and agonists like monophosphoryl lipid A (MPLA), have been shown to enhance the expression or activation of TLRs under specific conditions. Statins are known to modulate TLR2 and TLR4 expression, restoring immune balance by reducing inflammation and improving macrophage responsiveness [36]. Similarly, MPLA, a TLR4 agonist, can fine-tune macrophage activation and improve their ability to respond to pathogens, as demonstrated in preclinical models [37]. These approaches could help counteract the immune dysfunction caused by hyperglycemia by boosting TLR-mediated pathogen recognition and downstream signalling pathways.

Additionally, adjunct therapies targeting oxidative stress could complement TLR-based strategies. Hyperglycemia-induced reactive oxygen species (ROS) overproduction damages cellular components and impairs macrophage function. Antioxidants such as N-acetylcysteine (NAC) or vitamin C may help reduce oxidative damage, synergistically enhancing macrophage activity when combined with TLR-targeting drugs [38]. By addressing both TLR dysregulation and oxidative stress, these therapeutic strategies have the potential to restore effective immune responses in diabetic individuals. Future studies should investigate the combined effects of these interventions in clinical and experimental models of hyperglycemia-associated immune dysfunction.

In this study, we demonstrated that the increased susceptibility of human macrophages cultured in high glucose conditions to *Lb* infection may be linked to unaltered ROS production via TLR2 and TLR4 signalling post-infection. High glucose concentrations promote oxidative stress in macrophages, which likely hinders the generation of an effective immune response, particularly through these pattern recognition receptors (PRRs). These findings suggest that hyperglycemia-induced oxidative stress compromises macrophage functionality, leading to an insufficient defence against the parasite. Collectively, this

highlights a crucial pathway that may explain the increased vulnerability of diabetic patients to infections, specifically *Lb*. Understanding this mechanism opens potential therapeutic targets for managing infections in diabetic individuals. This study builds on existing research showing that high glucose levels disrupt immune responses through various mechanisms, including receptor downregulation and oxidative stress, further underlining the complexity of diabetic complications in immune defence.

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Disclosure statement

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