Inhibition of gamma-secretase activity without interfering in Notch signalling decreases inflammatory response in patients with cutaneous leishmaniasis

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ABSTRACT
Cutaneous leishmaniasis (CL) patients present an exacerbated inflammatory response associated with tissue damage and ulcer development. Increasing numbers of patients have exhibited treatment failure, which remains not well understood. We hypothesized that adjuvant anti-inflammatory therapy would benefit CL patients. The aim of the present study was to investigate the contribution of Notch signalling and gamma-secretase activity to the inflammatory response observed in CL patients. Notch signalling is a molecular signalling pathway conserved among animal species. Gamma-secretase forms a complex of proteins that, among other pathways, modulates Notch signalling and immune response. We found that Notch 1 cell receptor signalling protects against the pathologic inflammatory response observed in CL patients. Notch signalling, a gamma-secretase inhibitor that does not interfere with Notch signalling, was shown to decrease the in-vitro inflammatory response in CL. Our data suggest that JLK6 may serve as an adjuvant treatment for CL patients.

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Introduction
Leishmania braziliensis infection may lead to the development of cutaneous leishmaniasis (CL), the most prevalent clinical form of tegumentary leishmaniasis. CL lesions are characterized by an intense inflammatory infiltrate, high levels of pro-inflammatory cytokines TNF and IL-1β, and few parasites [1–6]. The exacerbated inflammatory response observed in these individuals is associated with ulcer development and disease progression [2,3,5,7]. Therapeutic failure in CL remains high, spanning from 30% to 70%, depending on the medication used for treatment and phase of the disease [8–11]. Thus, the search for novel adjuvant therapies, e.g. drugs that decrease inflammation without interfering in parasitic burden, is crucial.

The contribution of cytokines TNF and IL-1β in tissue damage arising from tegumentary leishmaniasis has been well-described [4,5,12,13]. The use of Pentoxifylline, a drug that decreases TNF production, as adjuvant therapy for mucosal leishmaniasis patients, the most severe form of tegumentary leishmaniasis, was observed to reduce time to healing and treatment relapse [14]. Recently, studies have documented the association of high levels of IL-1β with CL severity, and reported decreased levels upon cure [5,12,15,16]. Importantly, in humans, neither TNF nor IL-1β have been shown to participate in parasite killing, making the down-regulation of these cytokines an attractive approach for adjuvant therapy in tegumentary leishmaniasis, especially considering high reported rates of therapeutic failure [5].

The well-conserved signalling pathway mediated by Notch receptors controls a range of cell functions in mammals, including cell fate, cytokine production, cell activation and proliferation [17–20]. Notch signalling takes place through the binding of ligands, Delta-like or Jagged, to Notch receptors, which are then cleaved by the gamma-secretase protease complex. The Notch intracellular domain (NICD) translocates to the nucleus, binds to the mastermind transcriptional activator and other co-activators, and induces the transcription of Notch target genes [21]. Interestingly, some gamma-secretase inhibitors, such as JLK6, were not found to influence Notch signalling, unveiling a non-canonical Notch signalling pathway not dependent on gamma-secretase activity [22].
contribution of Notch signalling to mononuclear and T cell activation, as well as cytokine secretion, is well known [17–20,23–26]. Using an L. major-resistant mouse model of CL, it was documented that Notch 1 and 2 signalling is essential for IFN-γ secretion by CD4+ T cells in addition to parasite killing; however, the influence of this signalling on the secretion of pro-inflammatory cytokines was not investigated [27]. The expression of Notch receptors and Delta-like ligands was increased in CL lesions, particularly in patients that responded poorly to treatment, suggesting a role for Notch signalling in the promotion of an inflammatory response against CL [28]. To date, no functional analysis has been performed in human CL in an attempt to address the contribution of Notch signalling or gamma-secretase activity with respect to pro-inflammatory cytokine production, disease expression and parasite killing. To investigate this, we recruited CL patients and performed in vitro functional studies. Our results show that the expression of presinilin, a protein present in the gamma-secretase complex, was increased in CL lesions, and that the in vitro blockade of gamma-secretase activity decreased the inflammatory response without interfering in the parasite burden of mononuclear phagocytes.

Materials and methods

Subjects

This cross-sectional study was approved by the Institutional Review Board of the Professor Edgard Santos University Hospital Complex (HUPES-UFG) (protocol no. 25/12) and the Brazilian Commission of Ethics in Research (612.907). All subjects provided written informed consent. This study was conducted in accordance with the Declaration of Helsinki and subsequent revisions. A total of 36 CL patients were recruited from an area endemic for leishmaniasis – Corte de Pedra, Bahia-Brazil. All patients included had no mucosal disease or diabetes mellitus. Pregnant women, patients older than 60 and younger than 18 years old were also excluded. Diagnostic criteria consisted of the presence of an ulcerated skin lesion, with no evidence of mucosal involvement, and the detection of L. braziliensis DNA by PCR. The control group consisted of 5 healthy subjects (HS), living in a non-endemic area of the same state, without any reported exposure to Leishmania. All CL patients underwent clinical evaluations prior to beginning treatment.

RNA Sequencing

Unbiased RNA sequencing was performed on CL lesion and healthy subjects skin by our group, as previously described [29]. Raw sequence data is available on the Gene Expression Omnibus (GEO, accession # GSE127831).

Parasite cultures

An 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 [30]. Following isolation, parasites were cryopreserved in frozen nitrogen until use. The parasites selected for this study were not previously passed in liquid culture medium. After selection, parasites were expanded in Schneider’s medium (Sigma-Aldrich, St Louis, MO) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 1% L-Glutamine, penicillin (100 U/mL) and streptomycin (100 µg/mL) (Thermo fisher scientific, NY, USA).

Soluble Leishmania Antigen (SLA)

SLA was prepared from an isolate of L. braziliensis as previously described [31]. Briefly, promastigotes were re-suspended in lysing solution (Tris, HCL, EDTA and leupeptin), immersed in liquid nitrogen, and subsequently thawed at 37°C. After the freeze–thaw procedure, parasites were sonicated and then centrifuged at 14,000 × g. The supernatant was filtered and assayed for protein concentrations, tested for endotoxins using the Limulus amebocyte lysate test (Thermo fisher scientific, NY, USA), and used at a concentration of 5 µg/ml.

Peripheral blood mononuclear cell cultures and biopsies

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by Ficoll-Paque (GE Healthcare, Chicago, IL) gradient centrifugation (1450 RPM). After washing in saline, cell concentrations were adjusted to 3 × 10⁶ cells in 1 ml of RPMI-1640 (Thermo fisher scientific, NY, USA) supplemented with 10% FBS (Thermo fisher scientific, NY, USA), penicillin (100 U/ml) and streptomycin (100 µg/mL) (Thermo fisher scientific, NY, USA). PBMCs were dispensed into 24-well plates and incubated at 37°C under 5% CO₂ for 72 h in the presence or absence of SLA (5 µg/ml), anti-Notch-1 (20 µg/ml) (Thermo fisher scientific, NY, USA), anti-Notch-3 (20 µg/ml), DAPT (20 µM) and JLK6 (20 µM) (R&D Systems, Minneapolis, MS, USA). To determine cellular toxicity of gamma-secretase inhibitors (DAPT or JLK6), we performed a cell viability assay, MTT, and the concentration of 20 µM showed not to be toxic to PBMC.

Biopsies from L. braziliensis-infected patients and HS were performed using a 4-mm punch and cultured in complete RPMI media at 37°C, 5% CO₂ for 72 h in
the presence or absence of JLK6 (20 μM) (R&D Systems, Minneapolis, MS, USA). Supernatants were collected from PBMC and biopsy cultures and stored at −70°C until the time of cytokine quantification by ELISA (R&D Systems, Minneapolis, MS, USA), in accordance with manufacturer instructions. Non-stimulated cells did not produce relevant amounts of cytokines. Results are expressed in pg/ml.

**Monocyte cultures**

Monocytes were purified from PBMCs by negative selection using MACS columns (Miltenyi Biotec, CA, USA). Monocyte were prepared following a method previously described by our laboratory to yield a purity of 99%, then characterized by flow cytometry as CD14⁺CD3⁻ [32]. Briefly, PBMCs were separated using a Ficoll-Paque (GE Healthcare, Chicago, IL) gradient, placed in 24-well plates at the concentration of 5 million PBMCs/well in 1 mL of RPMI 1640 supplemented with 10% human AB serum plus penicillin (100 U/mL) and streptomycin (100 μg/mL), and after 5 h of incubation at 37°C, 5% CO₂, monocytes were separated by adherence to plastic. Next, cells infected or not with *L. braziliensis* (MOI 5:1), and stimulated or not with 20 μM of JLK6, RPMI-1640 (Thermo fisher scientific, NY, USA) supplemented with 10% FBS (Thermo fisher scientific, NY, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL) (Thermo fisher scientific, NY, USA) at incubation times of 37°C, 5% CO₂ for 2, 48 or 72 h. At each time point, infection rate and parasite burden were evaluated by optical microscopy.

**Results**

Gamma-secretase activity induces the cleavage of Notch receptors, which activates the Notch signalling pathway. To investigate the participation of Notch signalling components in CL we assessed unbiased gene expression in lesions from CL patients and HS. Our results show that the expression of genes involved in gamma-secretase activity, as *APH1A*, *APH1B*, *GSAP*, *NCSNTN*, *PSENEN*, *PSEN1*, and *PSEN2*, as well as those involved in inflammation (*NFKB1*, *NFKB2*, *RELA*, *TNF*, *IL6*, *IL1B*, *IL10*, *CXCL9*, and *GZMB*) and regulation of immune response, *IL-10*, were increased in CL lesion when compared to healthy skin (Figure 1(a)). The expression of most of genes involved in gamma-secretase activity positively correlated with genes involved in inflammation (Figure 1(b)).

To investigate the role of gamma-secretase activity in Notch signalling, we stimulated PBMCs cultures from CL patients with SLA in the presence of DAPT, a gamma-secretase and Notch signalling inhibitor, and then assessed levels of molecules associated with inflammation in CL (IL-6, IL-1β, TNF, CXCL9, granzyme B, and IFN-γ), and the regulatory cytokine IL-10. Inhibition of gamma-secretase activity by the non-selective inhibitor DAPT was observed to decrease the production of CXCL9 and IL-10 in PBMC cultures from CL patients (Figure 2).

Signalling through Notch-1 in macrophages increases the production of proinflammatory cytokines by these cells and Notch-3 is known to be up-regulated in inflammatory macrophages [23,33]. To examine the role of Notch-1 and -3 in the immune response against CL, we stimulated PBMCs from CL patients with SLA in the presence of Notch-1 and -3 neutralizing antibodies. While the neutralization of Notch-1 and -3 was not shown to effect TNF and granzyme B production, surprisingly, the blockade of Notch-1 increased SLA-induced IL-6, IL-1β and IL-10, and decreased CXCL9 production (Figure 3). These findings suggest that Notch-1 signalling inhibits the production of IL-1β and IL-6 in PBMCs, which would confer a benefit to CL patients. These results were unexpected, since available literature data show that signalling though Notch 1 receptor increases pro-inflammatory response by macrophages [23].

Notch signalling can also occur in the absence of gamma-secretase activity. As the inhibition of gamma-secretase produced an opposite effect than Notch-1 and -3 on the production of proinflammatory cytokines, we chose to employ a gamma-secretase inhibitor (JLK6) that does not interfere with Notch signalling [22]. Accordingly, PBMCs stimulated with JLK6 produced lower levels of SLA-induced TNF, IL-1β, IL-10, CXCL9 and granzyme B (Figure 4). To investigate the anti-inflammatory effects of JLK6 in lesion environment, we cultured cells from CL patients’ lesions in presence of JLK6. Our results show that inhibition of gamma-secretase activity with JLK6 decreased levels of IL-6, IL-1β, granzyme B and IL-10 in cultures of lesion cells (Figure 5).

Considering that the observed decreases in proinflammatory cytokines may favour pathogen growth, monocyte were infected with *L. braziliensis* and stimulated or not with JLK6 to investigate the ability of these cells to kill *L. braziliensis in vitro*. The addition of JLK6 increased parasite numbers at 48 h after infection, but did not affect the number of *Leishmania* killed by human macrophages after 48 h (Figure 6).

**Discussion**

Therapeutic failure rates are high in *L. braziliensis* transmission areas, reaching up to 70% depending on the clinical form of disease [9,10]. Since *L. braziliensis*-infected patients develop an exacerbated inflammatory response that leads to tissue damage, adjuvant therapy designed to decrease inflammation is desirable. For instance, patients with
mucosal leishmaniasis, the most inflammatory form of tegumentary leishmaniasis, benefit from the adjuvant treatment with pentoxifylline, a drug that down-regulates TNF production [14]. Interestingly, the use of pentoxifylline in combination with pentavalent antimony did not decrease time to cure or relapses in CL, suggesting that inflammatory mediators other than TNF may also play important in disease outcome [34]. Signalling through Notch receptors controls a variety of immunological process, as cell proliferation, CD4 T cell fate, macrophage activation and cytokines production [17,18,20,21,25–27]. Thus, interfering in Notch signalling as therapeutic approach may not be trivial since broad effects may be expected. Our current work investigated the role of Notch signalling on the production of inflammatory mediators in PBMCs and lesion cells from CL patients and found that JLK6, a gamma-secretase inhibitor, efficiently decreased the observed inflammatory response.

Signalling through Notch 1 in macrophages increases the production of proinflammatory cytokines by these cells [23]. A main finding of our work is that while the blockade of Notch 1 increased proinflammatory cytokine production, the inhibition of gamma-secretase activity by JLK6, a serine protease known to inhibit γ-secretase activity of other substrates but not of Notch, led to decreased levels of inflammatory mediators in cells cultures, supporting the notion that Notch receptor signalling is indeed beneficial to CL patients. It is already known that the Gamma-secretase complex interacts with a variety of substrates, including Notch signalling, and that the JLK6 compound does not directly inhibit the presenilin-dependent gamma-secretase complex; however, the

Figure 1. CL patients exhibit high abundance of components of gamma-secretase complex and inflammatory response genes expression in active lesions. (A) Unbiased RNASeq was performed on skin from 7 Healthy Subjects and lesion from 21 CL patients. Heatmap columns and rows represent each individual and gene, respectively. Heatmap colour reflects z-scores of gene abundance across samples. (B) Gene expression from gamma-secretase complex correlates with the inflammatory response in active lesions. Data from RNASeq (21 CL lesions) was used for correlation matrix between components of gamma-secretase complex and NFKB1, NFKB2, RELA, TNF, IL6, IL1B, IL10, CXCL9, and GZMB genes. Pearson’s test was used for correlation statistical analysis and p value is represented according to the size of the circles; *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.
underlying mechanism behind the action of JLK6 is not completely understood. Gamma-secretase inhibitors have been tested in clinical trials. While in T-cell acute lymphoblastic leukemia/lymphoma the use of Gamma-secretase inhibitor was well tolerated, in Alzheimer patients used to decrease the production of Amyloid Beta precursor protein, which is involved in the pathogenesis of this disease, problems with tolerability and side effects have been reported [35,36]. Since most clinical trials have shown that the systemic administration of gamma-secretase inhibitors is associated with severe side effects, mainly in the intestinal tract, the use of a topical formulation of JLK6 treatment would be a safer option in the case of CL.

Among the desired effects of JLK6 on the immune response of CL patients, a decrease in the production of granzyme B was observed. Cytotoxicity has been shown to contribute to the pathogenesis of CL, and granzyme B, produced mainly by NK cells and CD8+ T lymphocytes, was previously shown to induce the production of proinflammatory cytokines in CL patients [12,37]. Moreover, we observed discrepancies in the ability of JLK6 to

Figure 2. Non-selective gamma-secretase inhibitor (DAPT) decreases inflammatory-associated proteins production from CL patients in response to *Leishmania* antigens. PBMC from CL patients (n = 12) were cultured in presence or absence of SLA (5 µg/mL) and DAPT (20 µM) for 72 h. The levels of IL-6, IL-1β, TNF, IL-10, CXCL9, and granzyme B were determined in culture supernatants, by ELISA. The black line on the violin plot represents the percentile 50th and the dashed lines, 25th and 75th percentiles, respectively. Statistical analyses were performed using the Wilcoxon test **P < 0.01 and ***P < 0.001.

Figure 3. The neutralization of Notch 1 receptor increase production of proinflammatory cytokines from CL patients. PBMC from CL patients (n = 8) were cultured in presence or absence of SLA (5 µg/mL), anti-Notch 1 (20 µg/mL) and anti-Notch 3 (20 µg/mL) for 72 h. The levels of IL-6, IL-1β, TNF, IL-10, CXCL9, and granzyme B were determined in culture supernatants, by ELISA. The black line on the violin plot represents the percentile 50th and the dashed lines, 25th and 75th percentiles, respectively. Statistical analyses were performed using the Wilcoxon test *P < 0.05 and **P < 0.01.
modulate immune response in PBMCs versus cells from lesion (e.g. CXCL9 and TNF). These differences may be due to different cell number, composition and state of activation of these cells between both sites. Also, since these individuals have open skin lesions, it cannot be ruled out the interference of other pathogens (i.e. bacteria) in the immune response at lesion site. Finally, the presence of the regulatory cytokine, IL-10, allows parasite multiplication within macrophages \[38\]. Here the use of JLK6 in *Leishmania*-infected monocyte-derived macrophages decreased IL-10 levels, what may improve parasite killing. However, at time point of 48 h after infection we observed increase in parasite numbers within macrophages. Future studies will be performed to investigate the effects of JLK6 in reactive oxygen species (ROS) overtime, as it has been shown that ROS production is an important mechanism to kill *Leishmania* in human macrophages \[39\]. Altogether, these data show that decreasing inflammatory response with JLK6 does not affect the ability of macrophage to kill *Leishmania in-vitro.*
In conclusion, our current work document the advantage of blocking gamma-secretase activity without interfering in Notch signaling, making JLK6 a good candidate for adjuvant CL immunotherapy.

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Disclosure statement

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Data availability statement

These data is available at https://doi.org/10.6084/m9.figshare.13382723.v1

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