Metadados – Maurício Teixeira Nascimento

Tese de Doutorado

RESUMO

ESTUDO DE VIAS DE REGULAÇÃO DA RESPOSTA INFLAMATÓRIA EM PACIENTES COM LEISHMANIOSE CUTÂNEA CAUSADA POR *L. braziliensis.*

Pacientes com leishmaniose cutânea (LC) apresentam resposta inflamatória exacerbada associada a danos teciduais e desenvolvimento de úlceras. Nos últimos anos, foram observadas taxas mais elevadas de falha terapêutica ao antimoniato pentavalente, mas a razão subjacente permanece pouco compreendida. Nossa hipótese é que pacientes com LC apresentem uma diminuição dos mecanismos regulatórios, permitindo assim que a inflamação perdure e a úlcera se desenvolva. Os objetivos do presente estudo foi avaliar o papel da sinalização Notch, ativação do PPAR-y pela pioglitazona e as funções da PGE2 na resposta inflamatória de pacientes com LC causada por L. braziliensis. Nós inicialmente descobrimos que a sinalização via receptor Notch 1 protege os pacientes com LC contra a resposta inflamatória patológica, enquanto o composto JLK6, um inibidor seletivo da gama-secretase que não interfere na sinalização de Notch, demonstrou diminuir a resposta inflamatória in vitro, sem alterar a carga parasitária nos monócitos após 72 horas. Posteriormente, mostramos que ativação do PPAR-γ pela pioglitazona, alterou o perfil dos monócitos para M2, diminuiu a resposta inflamatória sem afetar a morte da L. braziliensis por monócitos de pacientes com LC. Por fim, descobrimos que o aumento da COX-2/PGE2 está associado à gravidade da doença e à falha terapêutica na LC. Além disso, a neutralização da COX-2 pelo NS398, um AINE seletivo, aumentou a capacidade dos macrófagos em matar a L. braziliensis, diminuindo assim a produção de citocinas inflamatórias. Juntos esses achados revelam as vantagens de inibir o complexo gamma-secretase com o composto JLK6 sem interferir na sinalização Notch, ativar o PPAR-y com a pioglitazona e inibir a síntese de PGE2 por meio da neutralização da COX-2 com composto NS398. Tornando esses três compostos fortes candidatos a terapia adjuvante da LC.

Palavras-chave: Leishmaniose cutânea; *L. braziliensis*; inflamação; falha terapêutica; Notch; Pioglitazona; PGE2; PPAR-γ

ABSTRACT

STUDY OF PATHWAYS OF REGULATION OF THE INFLAMMATORY RESPONSE IN PATIENTS WITH CUTANEOUS LEISHMANIASIS CAUSED BY L. braziliensis.

Patients with cutaneous leishmaniasis (CL) present an exacerbated inflammatory response associated with tissue damage and the development of ulcers. In recent years, higher rates of treatment failure with pentavalent antimoniate have been observed, but the underlying reason remains poorly understood. Our hypothesis is that patients with CL present a decrease in regulatory mechanisms, thus allowing inflammation to persist and ulcers to develop. The objectives of the present study were to evaluate the role of Notch signaling, activation of PPAR-y by pioglitazone and the functions of PGE2 in the inflammatory response of patients with CL caused by L. braziliensis. We initially discovered that signaling via the Notch 1 receptor protects patients with CL against the pathological inflammatory response, while the compound JLK6, a selective gamma-secretase inhibitor that does not interfere with Notch signaling, has been shown to decrease the inflammatory response in vitro, without change the parasite load in monocytes after 72 hours. Subsequently, we showed that activation of PPAR-γ by pioglitazone changed the profile of monocytes to M2, decreasing the inflammatory response without affecting the killing of L. braziliensis by monocytes from patients with CL. Finally, we found that increased COX-2/PGE2 is associated with disease severity and treatment failure in CL. Furthermore, neutralization of COX-2 by NS398, a selective NSAID, increased the ability of macrophages to kill L. braziliensis, thus decreasing the production of inflammatory cytokines. Together, these findings reveal the advantages of inhibiting the gamma-secretase complex with the compound JLK6 without interfering with Notch signaling, activating PPAR-y with pioglitazone and inhibiting the synthesis of PGE2 through neutralization of COX-2 with the compound NS398. Making these three compounds strong candidates for adjuvant therapy for CL.

Keywords: Cutaneous leishmaniasis; L. braziliensis; inflammation; therapeutic failure; Notch; Pioglitazone; PGE2; PPAR-γ

PAPER 1 - Inhibition of gamma-secretase activity without interfering in Notch signalling decreases inflammatory response in patients with cutaneous leishmaniasis

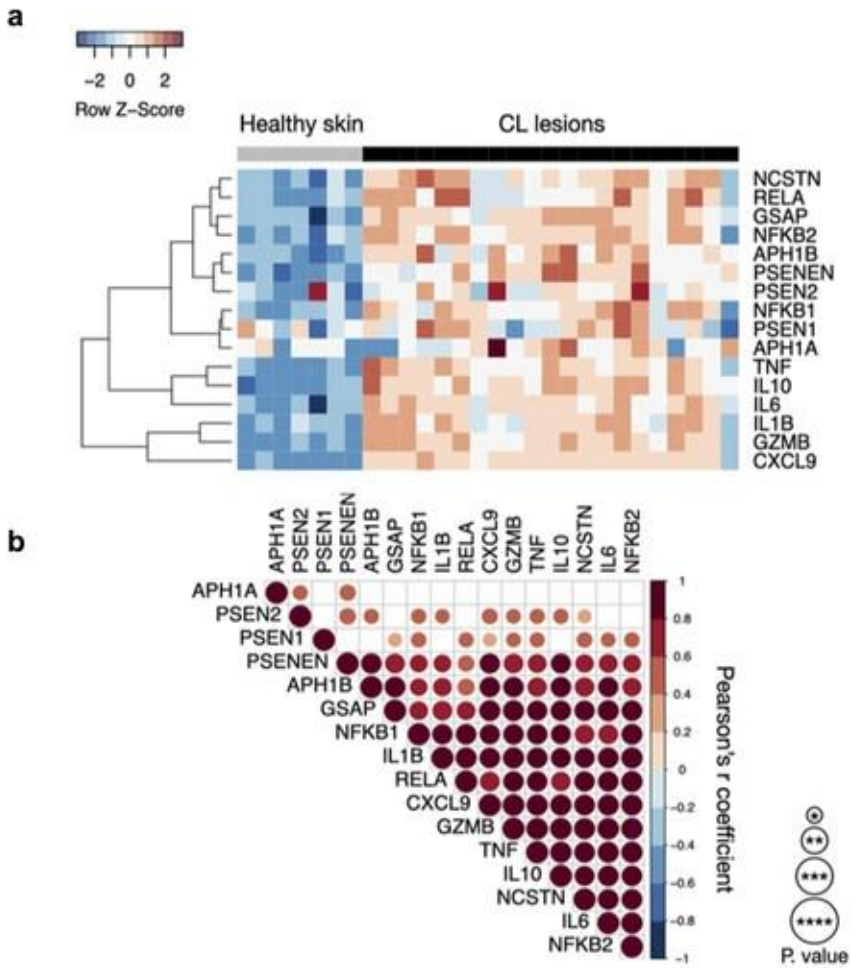

Figure 1. CL patients exhibit high abundance of components of gamma-secretase complex and inflammatory response genes expression in active lesions. (A) Unbiased RNASeq was performed on skin from 7 Healthy Subjects and lesion from 21 CL patients. Heatmap columns and rows represent each individual and 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.

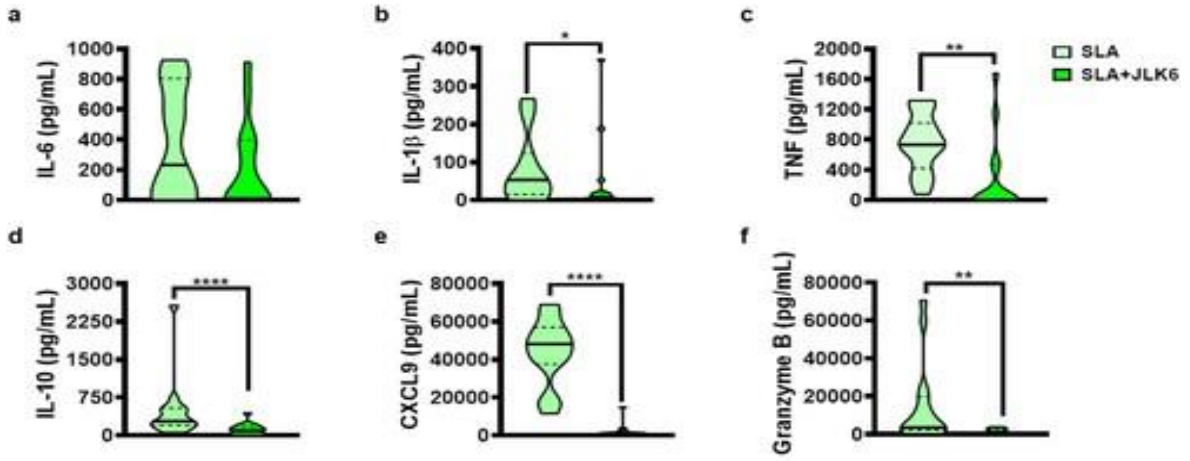

Figure 4. Selective gamma-secretase inhibitor (JLK6) decreases pro-inflammatory cytokine production from CL patients. PBMC from CL patients (*n* = 15) were cultured in presence or absence of SLA (5 ug/mL) and JLK6 (20 μM) for 72 h. The levels of IL-6, IL-1β, TNF, IL-10, CXCL9, and granzyme B were determined 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 *$P < 0.05$, **$P < 0.01$ and ****$P < 0.0001$.

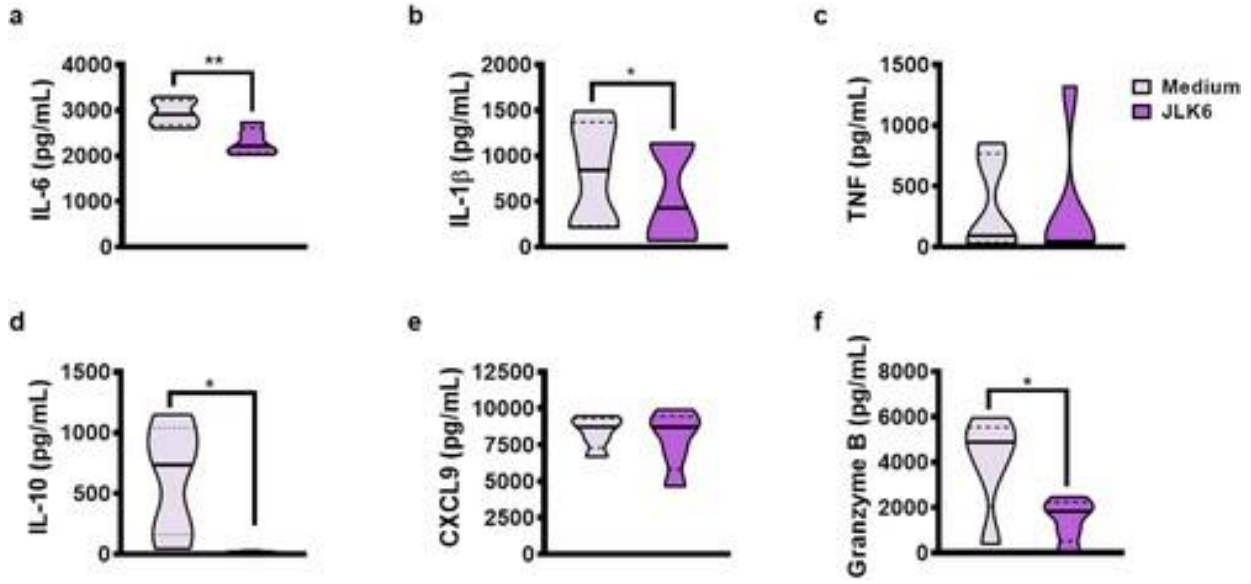

Figure 5. JLK6 downregulates pro-inflammatory cytokines production by lesion cells from CL patients. *L. braziliensis* lesions skin biopsies from CL patients ($n = 5$) were cultured in presence or absence of JLK6 (20 uM) for 72 h The levels of IL-6, IL-1β, TNF, IL-10, CXCL9 and granzyme B were determined 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 **$P < 0.01$.

Paper 2 - Pioglitazone, a Peroxisome Proliferator-Activated Receptor-γ Agonist, Downregulates the Inflammatory Response in Cutaneous Leishmaniasis Patients Without Interfering in *Leishmania braziliensis* Killing by Monocytes

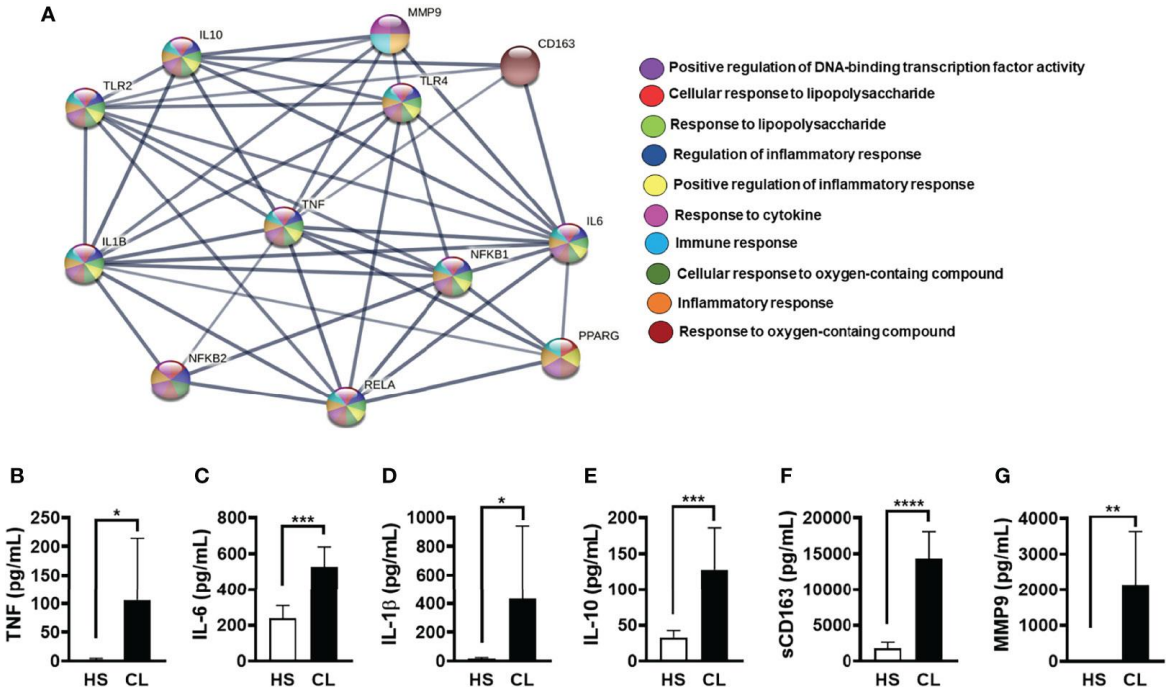

FIGURE 1 - STRING protein–protein interaction (PPI) network. (A) Enrichment analysis of the Gene Ontology (GO) and pathways revealed that a group of inflammatory proteins that participate in the pathogenesis of cutaneous leishmaniasis (CL) (IL-1β, IL-6, TNF, NFκB1, and RELA) were directly linked to the peroxisome proliferator-activated receptor-γ (PPAR-γ) protein. The lines show the interaction between the proteins and their thickness and the level of interaction between them. The analysis shows the enrichment value PPI ($p \leq 1.0–16$). Biopsies from CL patients ($n = 10$) and healthy subjects (HSs) ($n = 5$) were obtained with a 4-mm punch and cultured for 24 h. The levels of (B) TNF, (C) IL-6, (D) IL-1β, (E) sCD163 (F) MMP9, and (G) sCD163 were quantified by the ELISA technique. The

box represents the mean, and the line above the box is the SD. Statistical analyses were performed using the paired t-test. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

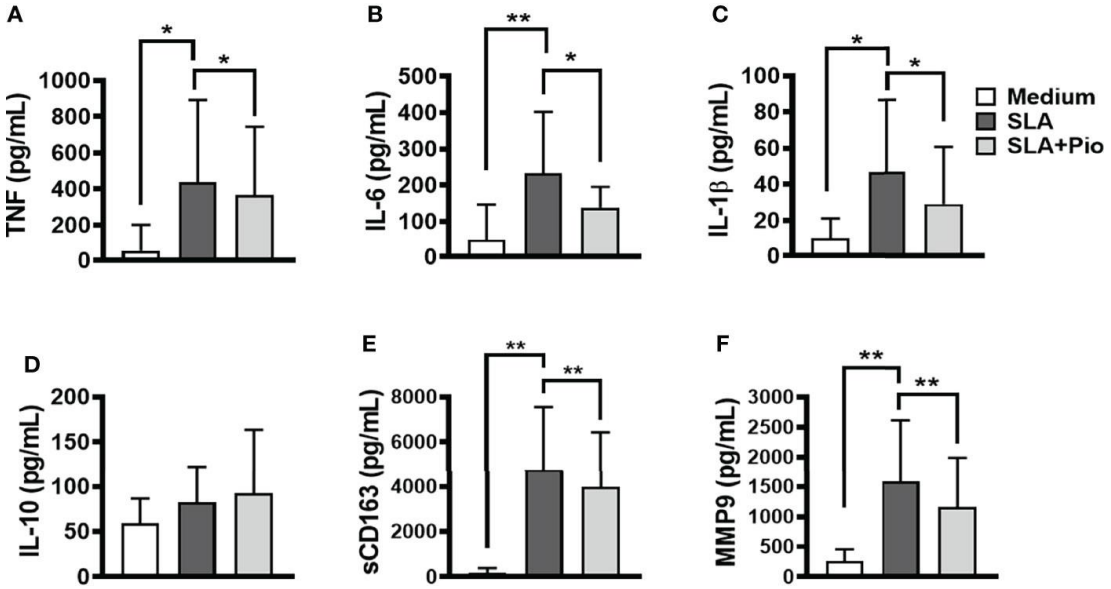

FIGURE 3 - Pioglitazone downregulates the production of inflammatory mediators induced by *SLA*. Peripheral blood mononuclear cells (PBMCs) from cutaneous leishmaniasis (CL) (n = 15) were cultured in the presence or absence of soluble *Leishmania* antigen (SLA) (5 µg/ml) or pioglitazone (1 µM) for 24 h. The levels of (A) TNF, (B) IL-6, (C) IL-1β, (D) IL-10, (E) sCD163 and (F) MMP9, were quantified by the ELISA technique. The box represents the mean, and the line above the box is the SD. Statistical analyses were performed using the ANOVA test. *p < 0.05, **p < 0.01, and ***p < 0.001.

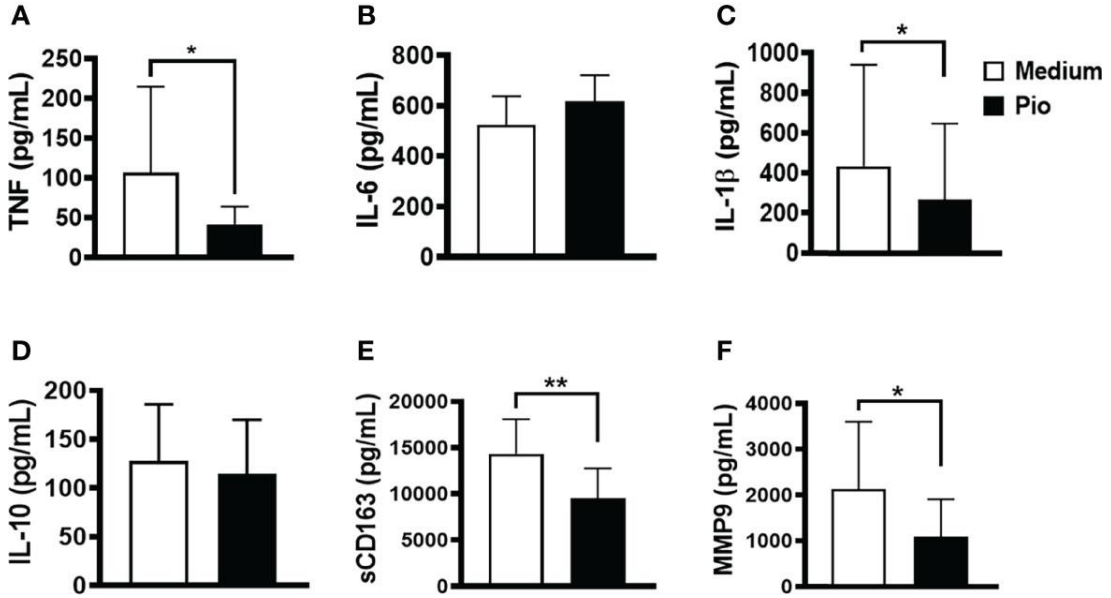

FIGURE 7 - Pioglitazone down-regulates the production of inflammatory mediators in cells from CL lesions. Biopsies from CL patients (n=10) and HS (n= 5) were obtained with a 4mm punch and cultured for 24 hours. The levels of (A) TNF, (B) IL-6, (C) IL-1β, (D) IL-10, (E) sCD163 and (F) MMP9 were quantified by the ELISA technique. The box represents the mean and the line above the box the standard deviation. Statistical analyses were performed using the Paired t test *P < 0.05.

Paper 3 - Prostaglandin E2 contributes to *L. braziliensis* survival and therapeutic failure in cutaneous leishmaniasis

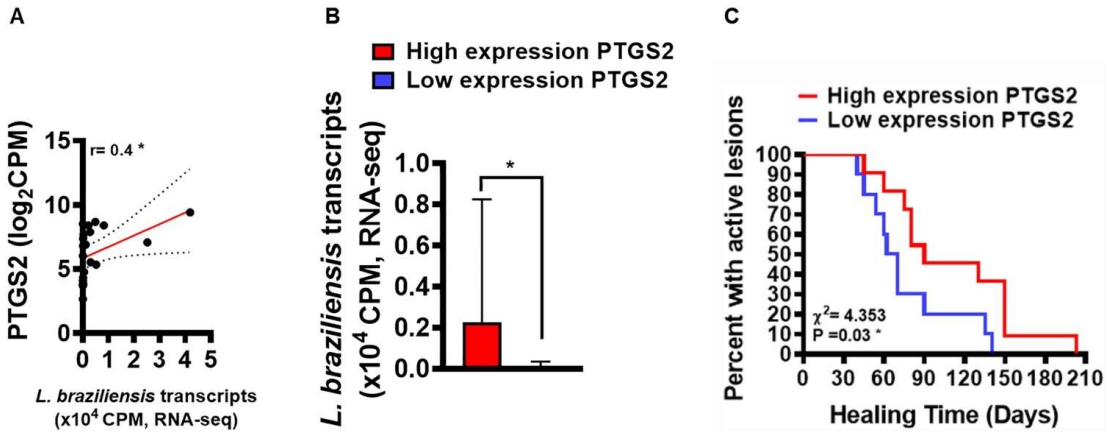

Figure 2. Increased PTGS2 gene expression at the lesion site is associated with parasite load and clinical outcome. RNAseq data (from 21 lesions) was used to investigate correlations between gene *PTGS2* and number of *L. braziliensis* transcripts. The mean expression of *PTGS2* was used as a cut-off point to determine high or low expression. *L. braziliensis* transcript number presented as median and interquartile range, with red bar indicating the group with high expression from *PTGS2* and blue bar low expression. Statistical analysis performed using Pearson's testing for correlations (A); the Mann–Whitney test was used for comparing parasite load in groups with high and low PTGS2 expression (B); Kaplan-Meier survival analysis of groups with respect to therapeutic failure (C) **P* < 0.05.

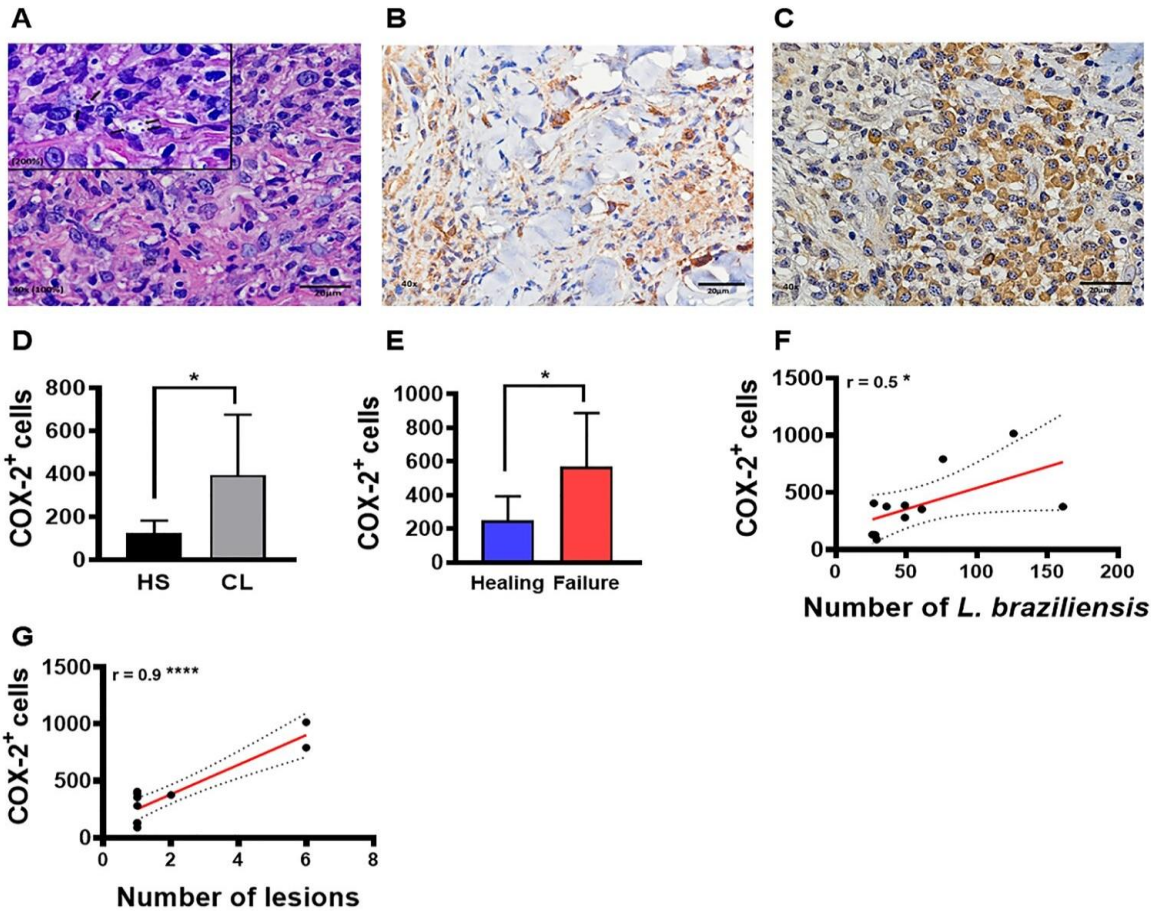

Figure 3. High frequency of COX-2$^+$ cells at the lesion site is associated with parasite load, disease severity and therapeutic failure. Lesion biopsies from CL patients ($n = 11$) and skin samples from healthy subjects ($n = 8$) were obtained using a 4 mm punch. Black arrows indicate the presence of *L. braziliensis* amastigotes in CL lesions (A), cells immunostained with polyclonal antibody anti-COX2 in healthy skin (B) and CL lesions (C), respectively. COX-2$^+$ cell frequency presented as mean and standard deviation: black bar indicates healthy skin while grey bar

represents CL lesions (D). Blue bar indicates patients that evolved to cure, while the red bar patients those that failed therapy (E) Correlation between number of amastigotes and frequency of COX-2^{+} cells (F), and correlation between number of lesions and frequency of COX-2^{+} cells (G). Statistical analysis performed using the Unpaired t-test to compare COX-2^{+} frequency, with Pearson's testing used for correlation analysis; *$P < 0.05$, ****$P < 0.0001$.

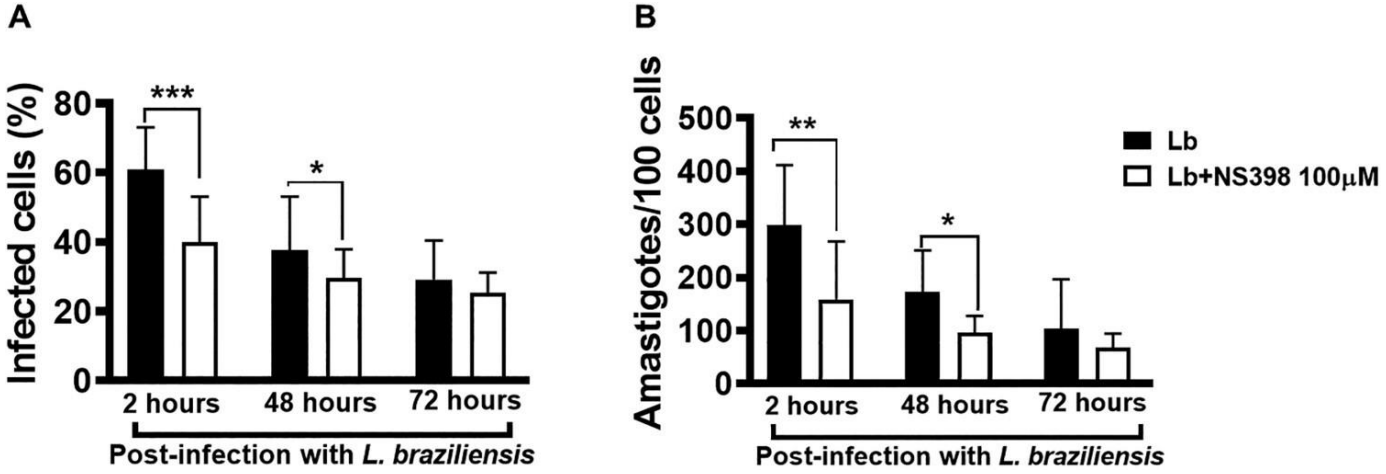

Figure 7. Neutralization of COX-2 increases *L. braziliensis* killing by macrophages obtained from CL patients. Monocyte-derived macrophages from CL patients ($n = 7$) were infected with stationary-phase *L. braziliensis* (5:1) and cultured in the presence or absence of NS398 (100 μM), a selective COX-2 inhibitor, for 2, 48 or 72 h. (A) Frequency of infected cells. (B) Number of amastigotes per 100 macrophages. Results presented as mean and standard deviation, with black bars indicating macrophages infected with *L. braziliensis,* and white bars macrophages infected with *L. braziliensis* treated with NS398. Statistical analysis performed using the Paired t-test; *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$.