# Expression of IL-1 Family Cytokines in Patients Before and After Cutaneous Leishmaniasis Cure

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**Research Article**

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# Abstract

Cutaneous leishmaniasis is an infectious disease that presents an immune response marked by the activation of lymphocytes and production of cytokines, including those of the IL-1 family, which act as an important trigger for the activation of an effector immune response. Despite this, inflammation exacerbation is sometimes also attributed to IL-1 cytokines, although some others down-regulate inflammation or produce Th2 responses, which need to be further clarified in the CL. Assessing the gene and protein expression of IL-1 cytokines associated with different immune response profiles in PBMCs from patients with active and healed lesions, this study demonstrated that stimulation by *L. braziliensis* positively regulates inflammatory and anti-inflammatory IL-1 cytokines, as IL-1α/β and IL-37, while there was a marked inhibition of IL-1Ra and IL-18 genes in patients treated with antimony, which perhaps contributes to the mechanisms of resistance that control *Leishmania* infection.


# Introduction

Cutaneous Leishmaniasis (CL) is a neglected infectious disease with a worldwide impact that mainly affects low-income people and has little investment in research and drug development. This clinical form is the most prevalent worldwide, and it is estimated that the regions of the Americas, Mediterranean, Middle East and Central Asia concentrate 95% of all cases [1, 2]. The infection is caused by protozoa of the genus *Leishmania*, which has several causative species, with *Leishmania braziliensis* having the greatest impact in the Northeast region of Brazil [3, 4]. Some limitations are observed in the available drugs, such as cost and different side effects [5, 6].

When symptomatic, the appearance and extent of skin lesions vary according to the species and immunological status of the patient [7, 8, 9]. Such participation of the immune response in the final manifestation of the disease is one of the important factors responsible for the wide clinical spectrum of CL. Furthermore, there is an important association between the immune response developed by the patient and the course of the infection, due to the intense inflammatory condition generated in response to the *Leishmania* infection, in an attempt to eliminate the parasite load [10, 11].

The IL-1 family cytokines, represented by 11 members, play a central role in immune responses against infections caused by pathogens, but their contribution to CL exacerbation is widely discussed and poorly understood in humans. They act by mediating the inflammatory response in a very similar way to IL-12 by inducing the generation of IFN-γ-secreting T lymphocytes. Thus, they tend to polarize the response to a Th1-type pro-inflammatory profile, as observed for the cytokines IL-1α and IL-1β, involved in the activation of macrophages, in addition to contributing to the activation of Th17 cells [12, 13, 14, 15]. Despite this, some members of the family are anti-inflammatory, such as IL-37, or act as antagonists of receptors that modulate inflammation driven by IL-1 itself. This makes this family complex regarding the roles played in immunopathogenesis in various pathological contexts, as well as in leishmaniasis. In this one, the controversies fall on the dichotomy “resistance and susceptibility” to *Leishmania*. This duality is strongly represented by IL-18, which can polarize Th1 or Th2 responses, by synergizing with other cytokines or

being influenced by the microenvironment [16, 17, 18, 19]. Furthermore, although poorly understood, the effectiveness of treatment with pentavalent antimonials seems to be associated with an effective activation of the patient's immune system [20].

In this study, we demonstrated an important modulation in the production of some IL-1 cytokines against *L. braziliensis*, a parasite with immunogenic potential that usually induces an intense inflammatory response, as well as the possible role of antimonial therapy in establishing an immune response less regulated and associated with resistance in patients who obtained clinical cure.

## Methodology

# Study population and ethical aspects

This study used blood samples from patients from different regions considered endemic for CL in Pernambuco. The patients were referred to a health unit, where a clinical diagnosis was made by a dermatologist. After that, blood samples were collected in partnership with the Reference Service in Leishmaniasis of the Aggeu Magalhães Institute (IAM-FIOCRUZ) to confirm the diagnosis with laboratory tests, which included direct research, PCR and indirect immunofluorescence

The following study groups were formed: 33 patients with active lesions, selected before treatment (BT) and 20 patients with clinical cure, based on complete re-epithelialization of the lesions, after undergoing treatment cycles with Glucantime® (called AT). The control group (CT) consisted of 10 healthy volunteers, living in non-endemic areas for cutaneous leishmaniasis and with no previous history of leishmaniasis. All of them were informed of the purpose of the study and blood samples were taken only after all participants and/or their legal guardians agreement to sign the “Free, prior and informed consent”. This research has been performed in accordance with the Declaration of Helsinki and with the Brazilian ethics law. The project was assessed by the Ethics Committee of the IAM-FIOCRUZ, receiving approval (CAEE: 11083812.7.0000.5190).

L. (V.) braziliensis **antigens fractions**

Promastigote forms (strain MHOM/BR/75/M2903) cultivated in vitro were expanded in Schneider's culture medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (SBF; Cultilab, Campinas, SP, Brazil) and 1% antibiotic (100IU/ml penicillin and 100 µg/ml streptomycin; Sigma, St. Louis, MO) until the exponential growth phase. The parasitic mass was then subjected to three washes with buffered saline (PBS-pH 7.2) by centrifugation at 800x g, for 15 minutes, at 4ºC. Subsequently, the cell pellet was resuspended in 750 µl of mild lysis buffer and 250 µl of protease inhibitor. The suspension was placed in eppendorf tubes, frozen and thawed 7 times in liquid nitrogen and a water bath until the cells were completely lysed. The suspension was then centrifuged at 4°C, 10,000 x g for 15 min. The supernatant, which corresponds to the soluble fraction of proteins, was collected and stored. Then, it was submitted to a protein dosage according to the method of Bradford (1976) [36] modified by Read &

Northcote (1981) [37], in addition to polyacrylamide gel electrophoresis (SDS-PAGE). After these procedures, the antigenic fractions were stored at –80ºC until use in cell culture assays.

# Obtaining peripheral blood mononuclear cells (PBMC)

Approximately 18 mL of blood was collected from each subject, then diluted 1:1 (v/v) in RPMI 1640 medium (pH 7.2), deposited in Ficoll-paque PLUS gradient solution (GE) and centrifuged for 35 min at 400 x g. Then, the layer of peripheral blood mononuclear cells (PBMC) was isolated, which underwent two cycles of washing with RPMI 1640 (pH 7.2) by centrifugation at 300 x g for 10 min. Then the cells were resuspended in RPMI 1640 containing 2 mM L-glutamine, 50 mg/L gentamicin sulfate, supplemented with 10% fetal bovine serum (FBS) (both Cultilab reagents, Brazil).

# Cell culture

After PBMC separation, $1x10^6$/ml cells were cultured in 5 ml polystyrene round bottom Falcon BD™ tubes, using RPMI 1640 medium supplemented with FBS for 72h (37°C/5% $CO_2$). For each control/patient, the cells were cultivated in two situations: one without stimulus (SE) and another that received the total antigen of *L. braziliensis* at a final concentration of 10 µg/ml. At the end of the culture, the tubes were centrifuged for 5 min at 300 x g. The cell culture supernatant was collected and stored at -20°C for cytokine dosage by ELISA.

# Cytokine assay from culture supernatant

To determine the quantification of IL-1α and β cytokines from the PBMC culture supernatant, Quantikines ELISA kits (RnD Systems, Minneapolis, MN, USA) were used following the manufacturer's recommendations for the assay. Their detection limits were 250 pg/mL The measurement of IL-37 was performed using the Uncoated ELISA Kit (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's recommendations for the assay. Its sensitivity is 31.3 pg/mL.

# Evaluation of mRNA expression by qPCR

The analysis started with the extraction of total RNA from the cultured PBMCs preserved in TRIzol™. Cells were vigorously homogenized for solubilization and release of genetic material. Then, RNA separation and precipitation were performed with the addition of chloroform and ice-cold isopropanol, respectively, by centrifugation at 10,000 x g for 10 minutes at 4ºC. The precipitate was washed with 1 ml of 75% ethanol, dried and resuspended in 20 µl of ultra-pure DEPC water. As quality control of the total RNA, the genetic material was run on a 1.2% agarose gel to visualize the integrity, in addition to dosing in NanoDrop™ 2000/2000c (Thermo Fisher Scientific, Waltham, MA, USA).

For the reverse transcription of RNA into cDNA, the TaqMan® Reverse Transcription Reagents kit (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA, USA) was used and the manufacturer's instructions were followed. The reaction had a final volume of 50 µl containing 1 µg of total RNA, 1x TaqMan RT Buffer, 5.5mM MgCl2, 500µM dNTPs Mix, 2.5µM random hexameters, RNAse inhibitor 0.4 U/µL, Multiscribe enzyme Reverse Transcriptase 3.125 U/Ml. The volume was made up with ultra-pure DEPC

water. The reaction consists of a cycle in the thermocycler, being ten minutes at 25ºC, thirty minutes at 48ºC and five minutes at 95ºC. After this step, the cDNA was stored at -20ºC until used in real-time PCR assays.

The mRNA levels were quantified using the TaqMan® Detection System through the QuantStudio™ 5 equipment and using the TaqMan® Universal PCR Master Mix kit (Thermo Fisher Scientific, Waltham, MA, USA). The probes used were: IL1A/FAM-MGB (Hs00174092_m1), IL1B/FAM-MGB (Hs01555410_m1), IL1RN/FAM-MGB (Hs00893626_m1), IL18/FAM-MGB (Hs01038788_m1) and IL37/FAM-MG001 (Hs00367201_m1). The reaction took place in a singleplex with a final volume of 20 µl, containing 1.5 µl of cDNA. Target genes were normalized by analyzing the 18S ribosomal RNA constitutive gene as endogenous control. The relative expression was calculated using Ct values, following the formula RQ = 2^-ΔΔCt, where ΔΔCt = ΔCt (control/calibrator group) – ΔCt (patient).

# Statistical analysis

Statistical analysis was performed using GraphPad Prism v.7 software (San Diego, CA, USA). The data were submitted to the normality test (Shapiro-Wilk), after that, using Mann-Whitney U test (non-parametric) and Student's T (parametric) according to the observed normality. All conclusions were taken at a significance level of 5% ($p < 0.05$ = statistically significant).

## Results

## • Clinical characteristics of patients with CL before and after treatment

The assessments were made from a total number of 33 patients diagnosed with CL and with active lesions before treatment (BT) and from 20 post-treatment patients who achieved clinical healing of the lesions after treatment with Glucantime® (AT). Summarized information about the patients' age, sex, number of lesions and scars, as well as the therapeutic regimen used in AT patients are shown in Table 1. The diagnosis of both groups was confirmed by a dermatologist and through laboratory tests, with PCR being the gold standard. The mean age of BT patients was higher than that observed in the AT group, in addition, there was no difference in the distribution of men and women between the groups (Table 1). The average time of evolution of the lesions, represented by the interval from the onset of symptoms until the patient's arrival at a health unit, did not differ significantly between the groups, and ranged from 15 days to 1 year in both groups of patients. The AT patients' blood collection was made, on average, about 250 days after the therapy, and the majority required 2 to 3 courses with Glucantime®, until there was complete re-epithelialization of the lesion, which is considered the criterion for clinical cure. These patients had a lower mean of lesions size compared to that observed in the BT group, which may be associated with response to treatment.

**Table 1**. General clinical assessment of patients included in the study. BT - before treatment, AT- after treatment, n/a-not analyzed, SD - standard deviation. $P < 0.05$.

| | BT | AT | P |
|---|---|---|---|
| | **N = 33** | **N = 20** | **-** |
| Age (mean, SD) | 42,06 ± 3,064 | 28,76 ± 3,476 | 0,007* |
| Male (%) | 56,67 | 43,33 | 0,5768# |
| Number of lesions/scars (mean, SD) | 2,45 ± 3,536 | 1,05 ± 0,2182 | 0,0049** |
| Evolution time (days average) | 69,73 | 60 | 0,6266** |
| Time before treatment (days average) | n/a | 250,24 | n/a |
| Average number of ampules (5 ml) | n/a | 2,5 | n/a |

# * Unpaired t test

# # Fisher's exact test

# ** Mann Whitney test

**• IL-37 is induced by** L. braziliensis **antigens together with IL-1 alpha and beta cytokines, in PBMC from patients even after clinical cure**

In vitro stimulation of PBMC was performed using total antigens obtained from *L. braziliensis*, at a concentration of 10 µg/ml, with the 72h stimulation time being the best condition tested and demonstrated in the results below (Fig. 1 and 2). Considering that the study's calibrator group is composed of healthy individuals who do not have the disease (CT), even in the absence of stimulation, patients BT and AT demonstrated higher levels of expression of the genes of the pro-inflammatory cytokines IL-1α (BT: p = 0.0226; AT: p = 0.025) (Fig. 1A) and IL-1β (Fig. 1C). Although IL-37 is considered an anti-inflammatory cytokine, the activation of its production depends on an inflammatory stimulus. As a result, we observed significantly higher levels of its mRNA expression in patients with active disease (p = 0.0001) compared to those who underwent treatment and who already have the hyper-inflammatory context resolved (Fig. 1E).

The increased expression of cytokines against antigenic stimulation by *L. braziliensis* is notorious in both groups of BT patients (*IL1A*: p = 0.0007; *IL1B*: p = 0.0039; *IL37*: p = <0.0001) and AT (*IL1A*, p = 0.0002; *IL1B*, p = 0.0012; *IL37*, p = 0.0005) (Fig. 1), indicating its ability to induce biased Th1 responses, but that seems to be compensated by the increased expression of *IL37*.

In addition to gene expression, we also confirmed IL-1β protein secretion by PBMCs from BT and AT patients even before there was stimulation (p = 0.0060 and 0.0358, respectively) (Fig. 1D). In response to

the antigen, the quantification of IL-1α (BT, p = 0.0008; AT, p = 0.0053) and IL-1β (BT, p = 0.0011; AT, p = 0.0055) increases considerably, whereas that of IL-37 was not significant (Fig. 1F).

- **Lower expression of IL-1 family cytokines associated with the Th2 profile of the immune response may reveal a less regulated response in patients after treatment**

Considering the heterogeneity of IL-1 cytokine functions, it is possible to observe that at least IL-18 and the antagonist IL-1Ra, family members that are associated with a more regulated immune response profile, often associated with susceptibility in the context of CL, had their respective genes inhibited after antigenic stimulation in BT and AT patients, being more significantly expressed in those who underwent the therapeutic scheme (*IL18*: p = <0.0001; *IL1RN*: p = 0.0171) (Fig. 2). IL-18 stands out in this study for being significantly less expressed in cured patients compared to active ones after a new stimulus, considering that they already had previous contact with the pathogen (p = 0.0004) (Fig. 2A).

# Discussion

The anthroponotic aspects of CL show characteristics of the disease such as its complexity, seen, for example, in the variations in the clinical forms and presentation of the lesions, which are mainly highlighted in the New World. Other aspects such as the individual's immune status, parasite load and the species of *Leishmania* also explain the broad spectrum of the disease in the region [11]. Among these, the activation of the immune system during the response to *Leishmania* infection is crucial not only for the outcome of the disease, but also seems to be associated with the effectiveness of the treatment with pentavalent antimonials. It is known, for example, that the use of antimonials improves the phagocytosis capacity of monocytes from infected patients, although it is not yet clear whether it is a direct or indirect mechanism of the drug [20, 21]. Although poorly understood, the role of some IL-1 cytokines has been shown to be important in the progression of CL pathogenesis in humans.

After three days of PBMC stimulation, we observed higher levels of *IL1A* expression and IL-1β protein quantifications in cells from patients with active and healed lesions, even in the absence of stimulation. Some data suggest that such cytokines are important for the activation of a Th1 response, CD4 T cells and IFN-γ production [22, 23], although they are also associated with dysregulation of the immune response [24, 25]. Our results suggest not only the stimulation capacity of *L. braziliensis* to direct an inflammatory response, but also that patients who underwent the treatment seem to present a resistant immune response after a new recognition of cells to *Leishmania* antigens, thus contributing to the cure and protection [26, 27]. This maintenance of immunological memory helps to explain the ability to produce inflammatory cytokines after stimulation by patients with CL, even after a long healing time [28, 29].

In the analysis of protein expression, the quantitation levels of IL-1β in both groups of patients before antigenic stimulation may indicate a persistent production during and after infection. On the other hand, lower IL-1α protein levels without stimulation can be observed since it is considered an “alarm” cytokine, which is restricted to the interior of the cell and is released after tissue damage and injury [30].

The inhibition behavior of *IL1RN*, a gene that encodes the protein that antagonizes the action of IL-1α and IL-1β agonists, is opposed to the elevation of such inflammatory cytokines. This may explain their strong role in the Th1 resistance response driven by *L. braziliensis* antigens, enabling them to play their role in inflammation and activate the immune cascades in response to the pathogen. Corroborating this, Dos Santos et al. (2017) [31] demonstrated in their study that *L. braziliensis* induces lower levels of IL-1Ra in a human monocyte lineage than *L. amazonensis*, evidencing a more inflammatory profile of this species.

These results also showed that, after restimulation, cells from patients who had CL and underwent the treatment expressed significantly less *IL1RN*, indicating that the rate of bioactive IL-1 cytokines capable of binding to the receptor is probably higher in these individuals [32]. The *IL18* significant reduction, seen mainly in the AT group, also contributes to the hypothesis that the absence of IL-18 is associated with attenuation of LC progression, perhaps due to the increase in the soluble IL-18 inhibitor receptor (IL-18BP), a target yet to be investigated in CL [27]. Although there are still no studies evaluating IL-18 in *L. braziliensis* infection, much less in humans, its role in CL associated with lesions and greater severity in mice has already been observed [19]. Furthermore, our data may indicate that the *IL18* transcriptional activity decrease may be a biomarker that signals a good prognosis and response to treatment, being a cytokine sensitive to therapy with Glucantime®.

On the other hand, we see evidence that there is a balance of the inflammatory response owing to the induction of *IL37* expression in response to *L. braziliensis*, whose protein product is known to inhibit the production of cytokines such as IL-1α, IL-6 and TNF, in addition to its production being dependent on an inflammatory stimulus [33, 34]. Although IL-37 secretion was not significantly detected in stimulated PBMCs, which can be explained by the marked presence of this protein in the cytoplasm of these cells [35], the increase in the number of copies of its mRNA provides clues that it plays its anti-inflammatory role in the pathogenesis caused by *L. braziliensis.* This data may be promising for therapeutic approaches or for use as a biomarker that signals exacerbated inflammatory activity.

Thus, we observed an important regulation of the production of IL-1 family cytokines against *L. braziliensis*, a parasite with immunogenic potential aimed at inducing an intense inflammatory response, as well as the role of antimonial therapy in the attenuation of IL-18, which appears to contribute to lesion healing. Our findings contribute in elucidating the participation of the IL-1 family and the still little-known role of IL-18 in CL susceptibility, as well as the regulatory role of IL-37 in response to *Leishmania*, which was not yet known.

# Declarations

### Author contributions

E. A. S. A., M. K. A. C. and A. A. S. performed the experiments, E. A. S. A., M. C. A. B. C. and V. R. A. P. conceived, conducted the experiments and analysed the results. All authors reviewed the manuscript.

### Additional information

The authors declare no competing interests.

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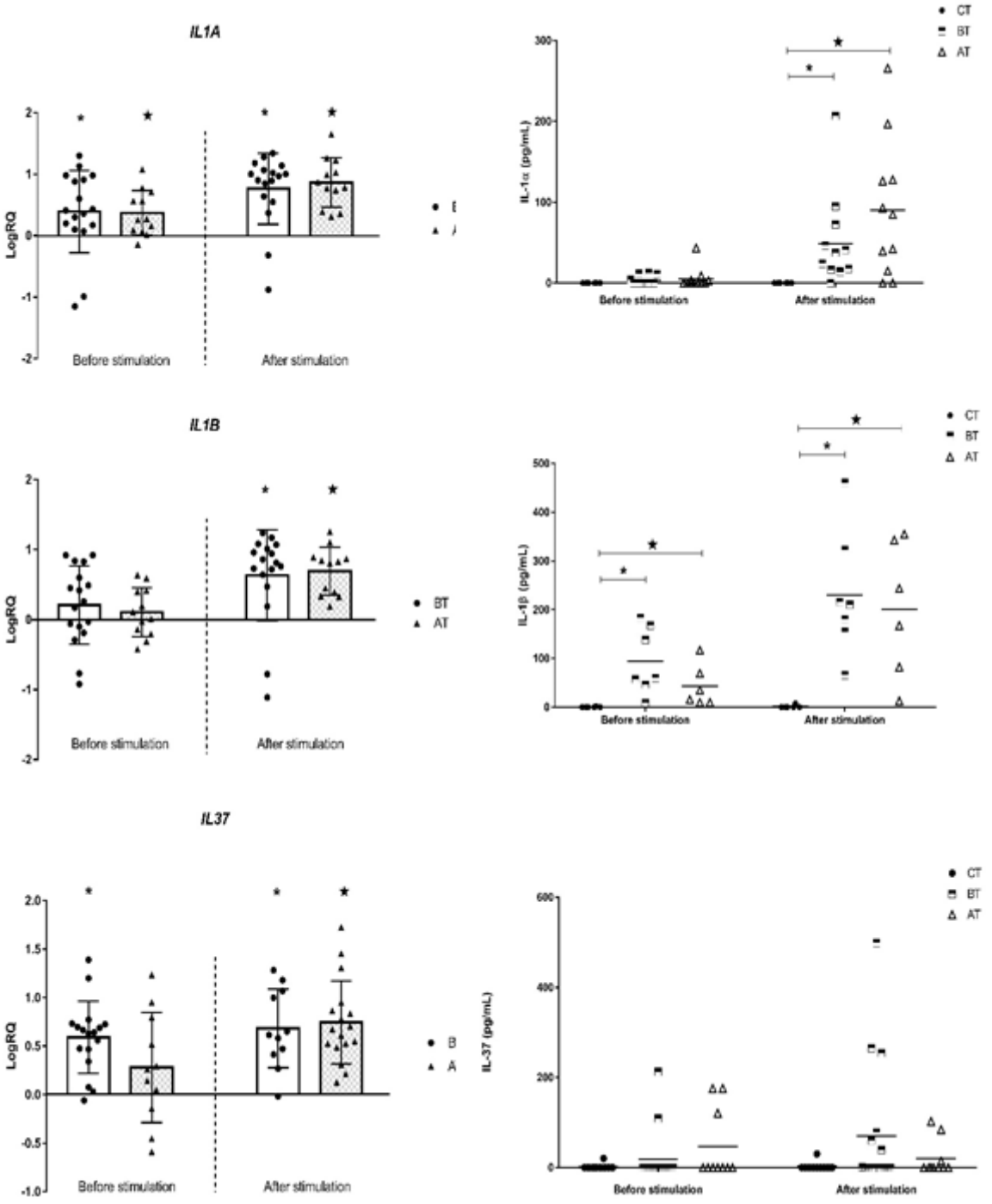
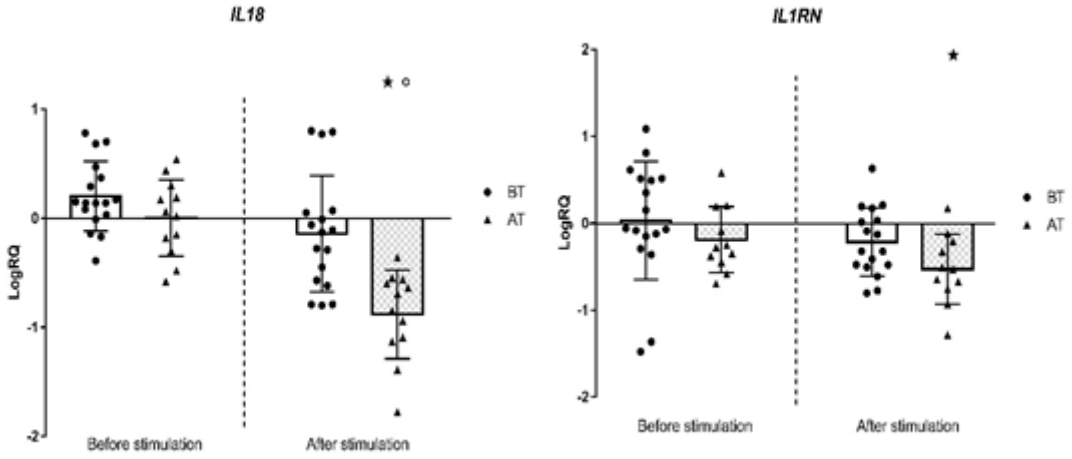
# Figures



## Figure 1

In vitro evaluation of gene and protein expression of IL-1α (A and B), IL-1β (C and D) and IL-37 (E and F) before and after 72h of stimulation with total antigen (10 µg/ml) of L. braziliensis in PBMCs from patients with CL before and after treatment, using a group of healthy individuals as calibrator. The results are shown with mean values + SD and are considered significant when p is less than 0.05, represented by the symbols: * = difference between CT x BT; □ = difference between CT x AT.




## Figure 2

In vitro evaluation of IL18 (A) and IL1RN (B) gene expression before and after 72h of stimulation with total antigen (10 µg/ml) of L. braziliensis in PBMCs from patients with CL before and after treatment, using a group of healthy individuals as a calibrator. The results are shown with mean values + SD and are considered significant when p is less than 0.05, represented by the symbols: □ = difference between CT x AT; ° = difference between BT x AT.