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Original Article

Immunoregulatory effects of soluble antigens of *Leishmania* sp. in human lymphocytes *in vitro*

Efeitos imunoregulatórios de antígenos solúveis de *Leishmania* sp. em linfócitos humanos in vitro

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Abstract

The clinical manifestations of cutaneous leishmaniasis (CL) depend not only on the infecting species involved, but also on the immune response of the individual. Although not yet well understood in humans, parasite survival and persistence are related to the cytokine profile and T cell proliferation, with the Th1 profile being related to cure, and the Th2 profile to disease progression. Considering the need for studies focused on the species with the highest circulation in the state of Amazonas, this study aimed to analyze the immunoregulation stimulated by soluble antigens (SLAs) of *Leishmania (L.) amazonensis* and *Leishmania (V.) guyanensis* in human lymphocytes *in vitro*, in order to understand the immune response of patients with CL. Lymphoproliferation was evaluated against stimuli of SLAs from *L. amazonensis* (100 µg/mL), SLAs from *L. guyanensis* (100 µg/mL) and phytohemagglutinin (10 µg/mL) using a BrdU Cell Proliferation ELISA kit after 72 h of incubation. Quantification of the cytokines IL-1b, IL-6, IL-8, IL-10, IL-12 and TNF was performed using the BD™ cytometric bead array human Th1/Th2/Th17 cytokine kit. Our results demonstrated that soluble antigens from *L. amazonensis* and *L. guyanensis* stimulated the lymphoproliferation of PBMCs from patients primo-infected with CL. Among the cytokines dosed, the highest concentrations were of IL-6 and IL-8, thus demonstrating that the soluble antigens evaluated are capable of inducing regulatory mechanisms.

Keywords: neglected diseases, American Tegumentary Leishmaniasis, immunology, enzyme-linked immunosorbent assay.

Resumo

As manifestações clínicas da leishmaniose cutânea (LC) dependem não apenas da espécie infectante envolvida, mas também da resposta imune do indivíduo. Apesar de ainda não bem esclarecido em humanos, a sobrevivência e persistência do parasito estão relacionados ao perfil de citocinas e proliferação de células T, relacionando-se a cura ao perfil Th1, e a progressão da doença ao perfil Th2. Considerando a necessidade de estudos voltados para as espécies de maior circulação no estado do Amazonas, este trabalho teve como objetivo analisar a imunorregulação estimulada por antígenos solúveis (SLAs) de *Leishmania (L.) amazonensis* e *Leishmania (V.) guyanensis* em linfócitos humanos *in vitro,* afim de compreender a resposta imune de pacientes com LC. A linfoproliferação foi avaliada frente a estímulos de SLAs de *L. amazonensis* (100 µg/mL), SLAs de *L. guyanensis* (100 µg/mL) e *Phytohemagglutinin* (10 µg/mL) utilizando *BrdU Cell Proliferation ELISA Kit* após 72h de incubação. A quantificação das citocinas IL-1b, IL-6, IL-8, IL-10, IL-12 e TNF foi realizada por BD™ Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine Kit. Nossos resultados demonstraram que os antígenos solúveis de *L. amazonensis* e *L. guyanensis* estimularam a linfoproliferação de PBMCs de pacientes primoinfectados com LC. Entre as citocinas dosadas, as de maiores concentrações foram de IL-6 e IL-8, demonstrando que os antígenos solúveis avaliados são capazes de induzir mecanismos regulatórios.

Palavras-chave: doenças negligenciadas, Leishmaniose Tegumentar Americana, imunologia, ensaio de imunoabsorção enzimática.

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1. Introduction

The leishmaniases are anthropozoonoses that comprise a group of neglected diseases and are caused by intracellular protozoa of the genus *Leishmania* (*Kinetoplastida, Trypanosomatide*) (Rocha et al., 2020; Vasconcelos et al., 2018). In Brazil, cutaneous leishmaniasis (CL) presents a great diversity of etiological agents, reservoirs and vectors, which makes it difficult to control (Brasil, 2017). In the Amazonas state, the most common species are *Leishmania (Viannia) guyanensis, Leishmania (Viannia) naiffi* and *Leishmania (Leishmania) amazonensis* (Chagas et al., 2021; Figueira et al., 2008; 2014).

Regarding the clinical manifestations of CL related to species that circulate in the northern region of Brazil, *L. amazonensis* is the cause of the diffuse form of the disease. It is a rare but severe form that is characterized by nodules and lesions in plaques that are diffusely infiltrated through the integument in patients anergic to antigens of *Leishmania* sp., and this makes it difficult to treat (Silveira et al., 2004; 2008; 2009; Guimarães-Costa et al., 2009). *L. guyanensis* causes single or multiple ulcerated skin lesions, which can be caused by simultaneous bites of several infected female sandflies or by secondary lymphatic metastases (Brasil, 2017).

The diagnosis of CL is based primarily on anamnesis, clinical evaluation of lesions and knowledge about its epidemiology (Nascimento et al., 2022). For confirmation, direct examination is used, through scarification of the lesion, as well as through serological and histopathological methods, culture, inoculum in animals and polymerase chain reaction (Brasil, 2017; Cerutti et al., 2017). Despite being related to a series of serious and adverse side effects on the cardiovascular, renal, hepatic and pancreatic systems (Carvalho et al., 2019), for more than half a century (Carvalho et al., 2019), the therapy recommended by the Brazilian Ministry of Health has involved pentavalent antimonials (Sb^{5+}).

Due to its complexity, the immune response to infection in humans is not yet well understood; however, as far as is known, the clinical evolution of the disease involves a cooperation network between cytokines, co-stimulatory molecules and the T-cell profile. In general, the cure is associated with the Th1 profile and the Th2 profile is associated with the progression of the disease (Oliveira et al., 2021). In this context, immunity to *Leishmania* spp. depends on several factors, ranging from the species of the parasite to the genetic differences in the hosts, which, associated with the T-cell profile and the production of cytokines that act on the proliferation of the cell subtype, which are crucial to determine the clinical course of the disease (Silva, 2016).

In visceral leishmaniasis, which is the best studied model, patients have a high concentration of cytokines, and the interaction of immune profiles is observed in the active phase of the disease, called the cytokine storm, of types Th1, Th2, Th17 and regulatory T (Treg) cells (Duthie and Reed, 2014). In cutaneous leishmaniasis, the triggering of the disease is the result of the balance between Th1 and Th2 responses, associated with the modulation of the response by the production of cytokines (Costa-da-Silva et al., 2022). Resistance to the parasite is related to the development of the Th1 response and the production of pro-inflammatory cytokines such as IL-2, IL-12, IFN-γ and TNF-α, while susceptibility to infection is related to the development of the Th2 response and the production of cytokines such as IL-4, IL-5 and IL-13 (Maspi et al., 2016; Scott and Novais, 2016).

Regarding the cytokines secreted during the immune response, IL-1b has been described as essential in the control of infection by *Leishmania* sp. by inducing nitric oxide (NO) production in macrophages, via inflammasome activation (Lima-Junior et al., 2013). This is in addition to inducing the production of IL-6, involved in processes of regulation of innate and adaptive immunity and, together, the increase of IL-8, which modulates the recruitment of other cell types, initiating the inflammatory process (Santos et al., 2016; Mayadas et al., 2014). However, the profile of the human immune response to the predominant species in the Amazon region is still not well understood, which makes its recognition fundamental in order to understand the development of the infection.

Thus, in order to understand the immunogenicity of soluble antigens of *L. (L.) amazonensis* and *L. (V.) guyanensis* in the immune response, this study aimed to evaluate the lymphoproliferation and cytokine production of patients with cutaneous leishmaniasis before and after conventional treatment with Glucantime®.

2. Materials and Methods

2.1. Extraction of the soluble antigen

The soluble *Leishmania* antigens (SLAs) used in cell stimulation assays were obtained from parasitic mass of reference strains of *L. (V.) guyanensis* (MHOM/BR/1975/M4147) and *L. (L.) amazonensis* (IFLA/BR/1967/PH8) maintained in the cryopreservation bank of the National Institute for Amazonian Research (INPA). The parasites were cultured in complete RPMI medium with 10% inactivated fetal bovine serum (FBS) until reaching the stationary phase, observed under an inverted optical microscope.

The antigens were obtained by centrifugation (at 14,000 rpm) of the parasites in the medium for 15 minutes, followed by washing the sediment with PBS (phosphate-saline buffer). After the last wash, the pellet was resuspended in 0.9% saline and the protease inhibitor was added. Then, thermal shocks were performed with freezing in liquid nitrogen and manual defrosting, followed by centrifugation at 14,000 rpm for 30 minutes. The sediment was discarded and, by using the supernatant, the protein concentration was measured via spectrophotometry, using the wavelength of 595 nm, according to the Bradford method (Bradford, 1976). The SLAs were diluted to 10 µg/µL in RPMI and stored at -80 °C until the *in vitro* assays were performed.

2.2. Patients with cutaneous leishmaniasis and the control group

Thirty volunteers met the inclusion criteria and were invited to participate voluntarily in the study. This study

was approved by the Ethics Committee in Research with Human Beings (REC) at the Federal University of Amazonas (UFAM) - CAAE/UFAM: 29406319.2.0000.5020.

The participants included in this study were organized into three groups: before treatment - ten primo-infected patients diagnosed with CL, though not yet having started treatment; ten post-treatment patients with healed lesions and conventional treatment with Glucantime® having been completed; and the negative control - ten healthy individuals, with no history of CL, and residents of non-endemic areas of the metropolitan region of Manaus (Silva et al., 2021; 2023). Of the thirty volunteers, ten had already been treated (Post-treatment group) and ten received treatment after collecting biological material (Before treatment group) in a basic health unit located in the municipality of Rio Preto da Eva (3°07'06"S, 59°W), Amazonas, Brazil.

The primo-infected volunteers previously underwent a physical examination and scarification of the inner edge of the lesion. The collected material was swabbed on a slide and then the slide was stained with a rapid Panoptic Kit (Laborclin®) for the detection of amastigotes of *Leishmania* sp. and confirmation of the diagnosis for CL. The collected samples were inoculated in NNN culture media (Neal, Novy and Nicolle) for parasitological confirmation of the promastigote form of the parasite.

2.3. Stimulation of PBMCs in vitro

In vitro lymphoproliferation assays were performed with peripheral blood mononuclear cells (PBMCs) isolated from the blood of the volunteers, as described in Silva et al. (2021). The PBMCs were transferred to 96-well plates, organized in triplicates and exposed separately to *L. (L.) amazonensis* SLAs [100 µg/mL] and *L. (V.) guyanensis* SLAs [100 µg/mL] for sensitization and stimulation of the immune response. As a positive control of the lymphoproliferative response, phytohemagglutinin - PHA [10 µg/mL] was used and, as a negative control, unstimulated cells were used in RPMI medium.

The plates were incubated in an oven with CO_2 levels at 5%, 95% humidity for 72 hours at 37 ºC and, after 18 h of incubation, BrdU (5-bromo-2'-deoxyuridine) [10 mM mL^{-1}] was added. After 72 hours of incubation, the plates were centrifuged at 2,200 rpm for 15 minutes, and the culture supernatants were collected and stored at -80 ºC for cytokine dosing.

The lymphoproliferation assay was performed according to the procedures described in Silva et al. (2021), following the manufacturer's protocol for the BrdU Cell Proliferation ELISA kit (Biotrak, Amersham, UK). Lymphoproliferation was evaluated based on the quantification of BrdU incorporation, by means of the mean increase in optical density determined via spectrophotometry (absorbance at 450 nm).

2.4. Cytometric quantification of cytokines

The levels of cytokines IL-1b, IL-6, IL-8, IL-10, IL-12 and TNF were quantified using the BD™ cytometric bead array (CBA) Human Th1/Th2/Th17 Cytokine kit, following the manufacturer's protocol. These pro- and anti-inflammatory cytokines were selected according to the pattern of the immune response to infection by *Leishmania* spp. (Maspi, Abdoli and Ghaffarifar, 2016). The samples were acquired in a cytometer (BD FACSCanto II, BD Biosciences, San Jose, CA, USA), and the FCAP-Array V3 software (BD Biosciences, San Jose, CA, USA) was used for data analysis (pg/mL), evaluated according to the standard curves provided in the kits.

2.5. Statistical analysis

Prior to the analysis, the Shapiro-Wilk test of normality was performed. For comparisons between the groups of patients, Kruskall-Wallis and Mann-Whitney analyses were performed for comparisons between the antigens used, using the PAST statistical software version 4.0 (Hammer et al., 2001). Significance was defined based on p-values of <0.05 (*p<0.05, **p<0.01, ***p<0001, ****p<0.0001).

3. Results

In general, lymphocyte-mediated immunity to the stimuli of soluble antigens of *L. amazonensis* (p=0.06), *L. guyanensis* (p= 0.01) and the mitogen (PHA) (p=0.21) was expressed at high levels. The mean lymphoproliferation stimulated by *L. amazonensis* (see Figure 1A) in the volunteers of the group before treatment was 75.3%, after treatment it was 90.9% and, in healthy volunteers, it was 68.1%. The mean lymphoproliferation stimulated by *L. guyanensis* (see Figure 1B) in the volunteers of the before treatment group was 71.1%, after treatment it was 83.8% and in the healthy volunteers it was 75.3%. The antigen preparations of *L. amazonensis* induced a strong lymphoproliferative response (>60%) in 75% of the volunteers of the three groups evaluated. Only PBMCs from two healthy volunteers had a low lymphoproliferation rate (≥20%) (see Figure 1A and 1B).

The lymphoproliferative responses of the PBMCs from post-treatment volunteers were significantly stronger than those of the cells obtained from healthy controls and primo-infected (before treatment) patients after stimulation with *L. guyanensis* antigen preparations (see Figure 1B). In the *in vitro* assays, the SLAs from *L. amazonensis* induced higher levels of cell proliferation in the group that received treatment with Glucantime® (see Figure 1A).

When evaluating the concentrations of cytokines produced in the culture supernatants as a function of soluble antigens, the average level of SLA-stimulated concentration of *L. amazonensis* for IL-6 was 5,545 pg/mL (0-238,151 pg/mL), for IL-8, it was 3,789 pg/mL (0.79-133.53 pg/mL), for IL-1b, it was 492.0 pg/mL (0-15 312 pg/ml), TNF-α was 705.6 pg/mL (0-50, 175 pg/ml), IL-10 was 27.71 pg/ml (0-19,223 pg/mL) and IL-12 was 0.962 pg/mL (0-6, 050 pg/mL). The mean level of stimulation elicited by SLA of *L. guyanensis* for IL-6 was 8,524 pg/mL (0-238,151 pg/mL), for IL-8, it was 5,760 pg/mL (0.5-133,529 pg/mL), for IL-1b, it was 388 pg/mL (0-288,985 pg/mL), TNF-α was 13.01 pg/mL (0-572,245 pg/mL), for IL-10, it was 23.84 pg/mL (0-221,680 pg/mL) and IL-12 was 0.691 pg/mL (0-4,320 pg/mL).

The results of the statistical analysis showed that there was significance between the groups evaluated. The significant difference occurred between the groups

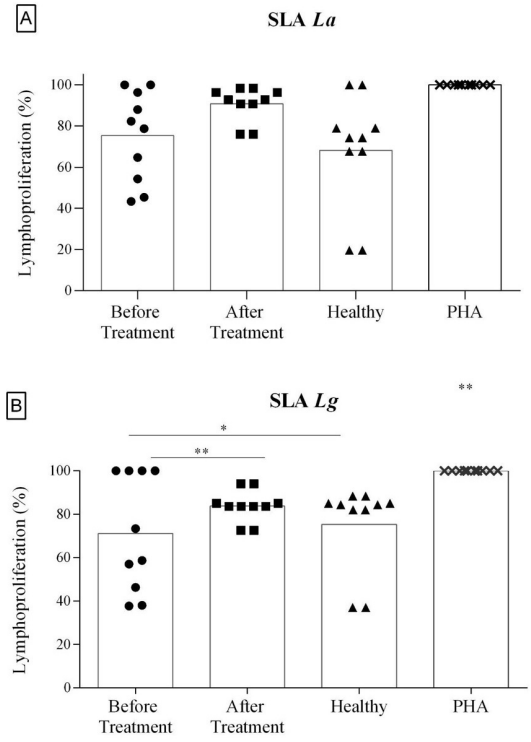

Figure 1. PBMC lymphoproliferation of participants evaluated in Rio Preto da Eva, Amazonas state, Brazil. A- soluble *Leishmania amazonensis* antigens (SLAs) and B- soluble *Leishmania guyanensis* antigens (SLAs) with primo-infected cutaneous leishmaniasis patients; after treatment - cutaneous leishmaniasis patients; and healthy people without cutaneous leishmaniasis; PHA: phytohemagglutinin. Vertical lines represent standard error. *Horizontal lines represent statistical significance between treatments ($p<0.005$).

regarding the production of IL-6 ($p=0.01$) and IL-1b ($p=0.04$) against the *L. guyanensis* antigen. Patients before treatment produced increased levels of IL-6 and IL-8 relative to post-treatment patients, in PBMCs sensitized with SLAs of both species (SLA-*La* and SLA-*Lg*). The remaining quantified cytokines (IL-10 and IL-12) had lower basal levels with lower concentrations in all groups of volunteers evaluated, with the exception of TNF-α dosage, which was relatively more evident in the group of healthy volunteers, in both sensitizations by soluble antigens (see Figure 2A,B).

In terms of concentration, IL-1b levels were higher for the primo-infected group whose PBMCs were stimulated by *L. guyanensis* antigens; however, the results were not statistically significant for this group. On the other hand, there was statistical significance for the post-treatment group in relation to the negative control ($p=0.01$), with lower concentrations for IL-1b.

4. Discussion

The cellular immune response is the main defense mechanism of the host against *Leishmania* sp., and the production of cytokines is crucial for the development of the immune response and control of the parasite (Silva et al., 2021). However, for this, efficient activation of T cells is necessary, as well as inflammatory immunoregulation to maintain the integrity of the host tissue (Antonelli et al., 2004).

Thus, resistance and susceptibility to infection by *Leishmania* sp. is associated with the level of expansion of Th1 and Th2 cells (Pirmez et al., 1993; Bacellar et al., 2002). The interaction between antigen and the T-cell receptor (TCR) culminates in the proliferation, differentiation and production of cytokines, which mediate the functions of effector T cells. As such, the lymphoproliferation assay is one of the models used to evaluate immunomodulatory activity (Zandonai, 2007; Pandima Devi et al., 2003).

In our results, it can be observed that the proliferation of lymphocytes sensitized by soluble antigens of *L. amazonensis* and *L. guyanensis* was high (see Figure 1). All clinical forms of CL are T-cell dependent and an exaggerated immune response to *L. amazonensis* antigens plays a crucial role in the pathogenesis of mucosal leishmaniasis (ML) (Bacellar et al., 2002).

Using total antigens of *L. braziliensis*, high lymphoproliferation was observed by Telino et al. (2006) at higher doses when compared to total antigen stimulation of *L. amazonensis*. The more positive stimulation for *L. braziliensis* was justified by the characterization of this species as the etiological agent of the patients treated. Regarding the results shown in this work, the soluble antigens of *L. amazonensis* and *L. guyanensis* induced high lymphoproliferation in all the *in vitro* assays and, although it is not known with which species the volunteers were infected, it is believed that the infection may have been caused by *L. guyanensis, L. amazonensis* or *L. naiffi,* since they are the predominant etiological agents in the city of Rio Preto da Eva, Amazonas (Figueira et al., 2014).

In relation to the cytokines, IL-6 was the cytokine that presented the highest concentrations in the supernatant of cells sensitized by soluble antigens of *L. amazonensis* and *L. guyanensis* (see Figure 2). IL-6 is a pleiotropic cytokine with anti- and pro-inflammatory properties, which mediates a number of physiological functions, including lymphocyte differentiation, proliferation and cell survival (Scheller et al., 2011). In this context, studies have shown different results regarding its protective and pathogenic activity in human and murine immunity against leishmaniases. It is recognized that IL-6 has a protective role against infection caused by *Leishmania (Viannia) donovani* and influences the ability of dendritic cells to regulate the development of $CD4^+$T cells (Bankoti and Stager, 2012).

In visceral leishmaniasis (VL), IL-6 deficient experimental animals may present susceptibility and higher parasitic load in target organs, due to the weak inflammatory response caused by the immunosuppressive effect of this cytokine (Murray, 2008; Silva, 2016). High levels of IL-6 are also associated with inhibition of TNF-α and Th1 responses in the early stage of *L. (L.) infantum* infection, triggering a generalized inflammatory response and, consequently, severity and death of patients with VL (Santos et al., 2016). Although IL-6 does not interfere with Th1 proliferation, this cytokine is related to the performance of the Th17 profile

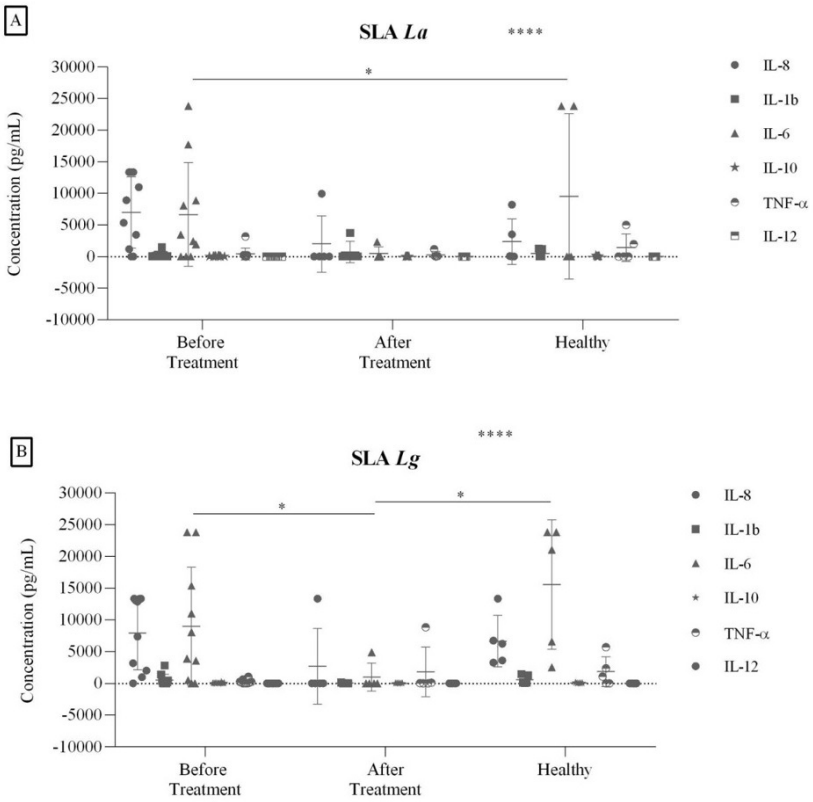

Figure 2. *In vitro* production of cytokines in participants evaluated in Rio Preto da Eva, Amazonas state, Brazil. A- soluble *Leishmania* antigens (SLAs) from *Leishmania amazonensis* and B- soluble *Leishmania* antigens (SLAs) from *Leishmania guyanensis* with primo-infected cutaneous leishmaniasis patients; post-treatment cutaneous leishmaniasis patients; and healthy people without cutaneous leishmaniasis; PHA: phytohemagglutinin. Vertical lines represent standard error. *Horizontal lines represent statistical significance between treatments ($p<0.005$).

at the beginning of infection, which demonstrates that it has an important role in modulating the neutrophil immune response and promotes protection against VL.

In our results with PBMCs stimulated by SLAs from *L. guyanensis*, we observed an increase in IL-6 production ($p=0.1435$) when compared with SLAs from *L. amazonensis*. The increase in IL-6 levels was observed in blood cultures of patients with the localized cutaneous form (LCL) and are associated with an immunopathogenic role (Costa, 2017). In the work of Silva et al. (2023), it was observed that the Cu(I) complex can increase cellular immune response against infection by *Leishmania* sp. given the high levels of cytokines IL-6 and IL-8 and strong induction of cell proliferation of PBMCs collected from patients with CL.

Regarding IL-8, the SLAs from *L. amazonensis* and *L. guyanensis* induced increased levels in the group of primo-infected patients when compared with the production of IL-6; however, there was no significant difference between the groups. *L. amazonensis* exerts an immunomodulatory effect on the immune response mediated by Langerhans cells (LC), inhibiting the production of IL-6 (Rodrigues, 2021). Inhibition of IL-6 by *L. amazonensis* has also been described by Craig et al. (2017), whose cultures of infected macrophages for 24 h showed inhibition of IL-6 production.

In addition, the levels of TNF-α were higher in the group of healthy volunteers using both soluble antigens (SLA-*La* and SLA-*Lg*), which is a common parameter due to the first exposure of PBMCs to *Leishmania* SLAs. TNF-α is considered pro-inflammatory cytokine and is associated with immunoprotection from and immunopathology of CL because, together with IFN-γ, it plays a crucial role in the elimination of *Leishmania* sp. through activation of macrophage activity for nitric oxide (NO) synthesis (Liew et al., 1997). In the PBMCs of the post-treatment group, TNF-α had very low concentrations (see Figure 2). It is noteworthy that the post-treatment volunteers received conventional treatment with Glucantime® before blood collection for use in this study; therefore, our findings corroborate those of Saldanha et al. (2012) and Muniz-Junqueira and Paula-Coelho (2008), whose *in vitro* experiments showed that TNF-α production by monocytes and neutrophils increases under exposure to the standard drug and decreases at the end of the treatment.

The mechanisms by which parasites suppress cytokine production are not known, but molecules contained in sandfly saliva, such as proteins, enzymes and prostaglandins, are indicated as inducers in the suppression of the inflammatory response and cytokine modulation

(Gillespie et al., 2000). Some drugs, such as Glucantime®, can also increase the production or suppression of cytokines, which increases the concentration of NO, IL-12 and TNF-α and decreases the level of IL-4 and IL-10 produced by macrophages of RAW 264.7 lineage infected by *L. infantum*, thus promoting an immunoprotective effect (Santos, 2018).

One of the pioneering studies with *L. major*, which is the best studied model at the immunological level, showed that infected monocytes induce the production of IL-8 and MCAF, but not IL-1b and TNF-α. It is suggested that the expression of chemokines may contribute to cell recruitment in lesions caused by *Leishmania* sp. (Badolato et al., 1996). With regard to IL-1b, in our results, the production of this cytokine occurred at low levels both against antigens of *L. guyanensis* and *L. amazonensis*.

Studies with experimental models have shown that the use of soluble antigens associated with antimony, antigenic preparations with or without adjuvants, and immunogenic molecules present in sandfly saliva can be effective in preventing an exacerbated infection, since pre-exposure through these antigens can favor the regulation of the immune response (Gillespie et al., 2000; Kamhawi, 2000; Machado-Pinto et al., 2002; Coler and Reed, 2005).

Analyzing the lymphoproliferative response to antigen stimulation, patients, before and after treatment, showed a high lymphoproliferative response (≥80%) and induction of increased levels of pro-inflammatory cytokines in the before-treatment patients. Thus, it has been shown that these soluble antigens induce cellular immune response, but that this response seems to be regulated after treatment, with a decrease in the production of these cytokines. This fact may be related to an efficient cellular immune response after stimulation of antigens, a necessary factor for maximum effectiveness of drug treatment and parasite control (Berger and Fairlamb, 1992). This relationship may favor the reduction of the parasitic load and, consequently, decrease the production of inflammatory cytokines that can cause exacerbated lesions.

5. Conclusion

In this study, we were able to conclude that the soluble antigens evaluated are capable of inducing regulatory mechanisms. However, in order to better elucidate the factors related to the clinical evolution of these patients, it is necessary to carry out further studies regarding the characterization of the species and the dosage of cytokines of different profiles. Understanding these induced mechanisms is essential in order to be able to propose new treatments that are capable of improving the quality of life of the patients and the prognosis of the disease.

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