Cellular immune response evaluation of cutaneous leishmaniasis patients cells stimulated with *Leishmania* (Viannia) *braziliensis* antigenic fractions before and after clinical cure

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**ABSTRACT**

American cutaneous leishmaniasis (ACL) is a disease where susceptibility or resistance is dependent on T cell response. This is characterized by an increased in CD4⁺ T cells, capable of inducing opposite disease profiles, and CD8⁺ T cells, that are related to immuno protection. We characterized T lymphocytes from patients before and after treatment, that spontaneously healed and controls, also evaluating their production of IL-10, IL-4, TNF-α and IFN-γ after stimulation with soluble/insoluble antigenic fractions of *Leishmania* (Viannia) *braziliensis*. We observed the production of suppressive cytokines in the early phase of leishmaniasis with significant presence CD4⁺ T cells, suggesting their connection with disease progression. After healing, the immune pattern observed was a type 1 response, what seems to be associated with cure and/or protection in the ACL. The results also showed that both fractions induced a specific immune response, contributing to the search for relevant antigens in this disease.

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1. Introduction

Leishmaniasis is considered an emerging and re-emergent disease, with an increase in its incidence in the last decades [1]. It has a global estimated prevalence of 12 million cases, with an estimated of 1.5–2 million new cases each year. At the present moment leishmaniasis occurs in 88 countries throughout Europe, Africa, Asia and America, and 350 million people are at risk of contracting the disease [2,3].

The American cutaneous leishmaniasis (ACL) is caused by different species of the genus *Leishmania*, and *Leishmania* (Viannia) *braziliensis* is the prevalent etiologic agent in Latin America, being also the prevailing agent in the Northeast region of Brazil and in the state of Pernambuco [4,5]. The clinical manifestations are broad and are determined by the species of leishmania, vector virulence factors and host immune response [3,6]. The pathogenesis is related to the interactions between the host innate and adaptive immune system, as well as the nutritional status and genetic factors. The disease may vary from asymptomatic forms to cutaneous and mucocutaneous ones [1,3,6].

In all ACL clinical forms, T lymphocytes are critical for cure and generation of a protective immune response, also being responsible for the disease persistence and pathology [7–9]. CD4⁺ and CD8⁺ T cells act producing biologically relevant cytokines for the activation of monocytes and macrophage. In human leishmaniasis, the type 1 immune response, with the production of IFN-γ, TNF-α and IL-12, has been associated with the infection control, by macrophage activation and parasite destruction [1,3]. On the other hand, cytokines like IL-4, IL-10 and TGF-β, considered of type 2, favor parasite multiplication, inhibiting NO production by IFN-γ activated macrophages [6,7,10]. These cytokines are also able to inhibit T cell differentiation into a type 1 profile and the subsequent production of IFN-γ and TNF-α [11]. Therefore, the quality of the immune response can influence disease susceptibility and development, with specific activation of T cells (especially multifunctional T cells) contributing to it [12]. However, even though there are studies that helped to clarify the immune mechanisms of disease development and pathology, no clear data is available about what immune response is necessary to control the disease and what is the difference between patients that develop the disease and achieve clinical cure (with or without treatment).

We examined in this study the CD4⁺ and CD8⁺ balance *ex vivo* and in vivo, and also the intracellular cytokine profile in patients’ cells after stimulation with the soluble and insoluble *L.* (*V.*) *braziliensis* antigenic fractions. The differences in the immune response and profile of patients before and after treatment, and also of...
individuals with lesions that spontaneously healed, were investigated together with the capacity of our antigens to elicit it, helping in the development of future vaccines and immunotherapies.

2. Methods

2.1. Study group

The patients of the present study were from different endemic municipalities of Pernambuco, Brazil. Seventeen patients were selected before treatment (BT), with a disease evolution around 1.6 months; 11 of them returned 6 months after treatment (PT) and 5 were patients that spontaneously healed (SH). The selection of these individuals was based on criteria such as: being more than 12 years old, having confirmed diagnosis by the CPqAM/FIOCRUZ Leishmaniasis Referee Center, being active lesion carriers when 12 years old, having confirmed diagnosis by the CPqAM/FIOCRUZ and 5 were patients that spontaneously healed (SH). The selection of these individuals was based on criteria such as: being more than 12 years old, having confirmed diagnosis by the CPqAM/FIOCRUZ Leishmaniasis Referee Center, being active lesion carriers when 12 years old, having confirmed diagnosis by the CPqAM/FIOCRUZ.

Patients selected before treatment were submitted to blood collection prior to chemotherapy treatment with Glucantime® (Sanofi-Aventis, Suzano, SP, Brazil). Patients that returned after treatment and that spontaneously healed had their blood collected after cicatrization of their lesions. Ten healthy individuals (CT) represented the control group from non endemic areas and without previous ACL infection. All of them signed the "Term of Free and Informed Consent". The CPqAM/FIOCRUZ Research Ethics Committee, Recife, Brazil approved the experimental protocols.

2.2. Leishmania antigenic factions

Promastigote forms of L. (V.) braziliensis (MHOM/BR/75/M2903), cultured in vitro, were expanded in Schneider’s medium (Sigma, St. Louis, MO) supplemented with 10% of fetal calf serum (Cultilab, Campinas, SP, Brazil) and 1% of antibiotics (100 UI/ml penicillin and 100 µg/ml streptomycin; Sigma, St. Louis, MO) until they reached the exponential phase. Afterwards, they were sedimented by centrifugation at 800 × g for 15 min at 4 °C and three times washed with phosphate-buffered saline (PBS; pH 7.2). Proteases inhibitors such as 0.1 mM methyl-phenyl-fluoride and 2 mM ethylendiaminetetraacetic acid (Sigma, St. Louis, MO), pepstatin A 0.001 M (Sigma, St. Louis, MO), were added right after ultrasonication (100 w/10 cycle/30 s with 2 pulses/s). The parasitic suspension was centrifuged at 10,000 × g for 10 min at 4 °C. The resultant supernatant was removed and submitted to a new centrifugation at 100,000 × g for 1 h at the same temperature. The resultant supernatant was the soluble antigenic fraction (SOF), while the sediment was the insoluble antigenic fraction (INS). Both were submitted to protein determination according to Brite et al. [13]. The antigens were stored at –20 °C until use.

2.3. Electrophoretic profile of soluble and insoluble antigens of L. (V.) braziliensis

The protein profile of the soluble and insoluble antigens of L. (V.) braziliensis were determined by a SDS (sodium dodecyl sulfate)–polyacrylamide gel electrophoresis. They presented proteins whose molecular weights varied between approximately 66 and 16 kDa compared to the standard molecular weight (Sigma, St. Louis, MO) (Fig. 1).

2.4. Obtaining the PBMC

Forty milliliters of peripheral blood in heparinized tube were collected, diluted in a proportion of 2:1 in PBS pH 7.2 and then added to a Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden) gradient. After centrifugation at 400 × g for 30 min at 20 °C a peripheral blood mononucleated cells (PBMCs) ring was obtained. The cells were washed twice with PBS pH 7.2 and submitted to centrifugation (300 × g for 15 min at 20 °C). PBMCs were resuspended with RPMI 1640 medium (Cultilab, Campinas, SP, Brazil) supplemented with 10% of fetal calf serum (Cultilab, Campinas, SP, Brazil) and 1% of antibiotics (100 UI/ml penicillin and 100 µg/ml streptomycin; Sigma, St. Louis, MO) until they reached the exponential phase. Afterwards, they were sedimented by centrifugation at 800 × g for 15 min at 4 °C and three times washed with phosphate-buffered saline (PBS; pH 7.2). Proteases inhibitors such as 0.1 mM methyl-phenyl-fluoride and 2 mM ethylendiaminetetraacetic acid (Sigma, St. Louis, MO), pepstatin A 0.001 M (Sigma, St. Louis, MO), were added right after ultrasonication (100 w/10 cycle/30 s with 2 pulses/s). The parasitic suspension was centrifuged at 10,000 × g for 10 min at 4 °C. The resultant supernatant was removed and submitted to a new centrifugation at 100,000 × g for 1 h at the same temperature. The resultant supernatant was the soluble antigenic fraction (SOF), while the sediment was the insoluble antigenic fraction (INS). Both were submitted to protein determination according to Brite et al. [13]. The antigens were stored at –20 °C until use.

Table 1

Test results of the study group and patient status.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Study group</th>
<th>MST(^a) (mm)</th>
<th>Direct microscopy</th>
<th>IFI(^b)</th>
<th>PCR(^c)</th>
<th>Number of Ampoules of Glucantime(^d)</th>
<th>Patients status</th>
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<tbody>
<tr>
<td>1</td>
<td>BT</td>
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<td>N/D</td>
<td>1</td>
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<td>SH</td>
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</table>

\(a\): positive; \(–\): negative; N/I: not informed; N/D: not done; BT: before treatment; PT: after treatment; SH: spontaneously healed.

\(^a\) Montenegro skin test.

\(^b\) Indirect immunofluorescence.

\(^c\) Polymerase chain reaction.

\(^d\) Number of Ampoules of Glucantime® used.

![Fig. 1. SDS–PAGE of soluble proteins (1 and 2) and insoluble (3 and 4) of L. (V.) braziliensis.](image-url)
SP, Brazil) and 1% antibiotic (100 UI/ml penicillin and 100 µg/ml streptomycin; Sigma, St. Louis, MO) and then counted in a Neubauer chamber using Trypan blue (Sigma, St. Louis, MO). The cells where then adjusted to a concentration of 4 × 10^6 per tube/ml.

2.5. Cell culture and flow cytometry

PBMCs (4 × 10^6 per tube/ml) were incubated with *Leishmania* soluble (SOL, 1.25 µg/ml) and insoluble (INS, 2.25 µg/ml) antigenic fractions (37 °C/5% CO₂), for 48 h, by means of previous standardization. Brefeldin A (10 µg/ml) (Sigma, St. Louis, MO) was added to all tubes 4 h prior to the end of the incubation period. After the incubation time, ethylene-diamine-tetra-acetic acid (EDTA, 20 mM) (Sigma, St. Louis, MO) was added to the culture and then incubated for 10 min. The cells where then washed with PBS with 0.5% bovine serum albumin (Sigma, St. Louis, MO) and 0.1% sodium azide (Sigma, St. Louis, MO), a solution called PBS-W, centrifuged (7 min, RT) and transferred to polystyrene tubes. The tubes contained monoclonal antibodies anti-CD4 or anti-CD8, both labeled with fluorescein isothiocyanate (FITC) (BD Bioscience, San Jose, CA), and the cells were incubated for 30 min at RT. Afterward the cells were fixed with 1% paraformaldehyde in PBS, washed by centrifugation with PBS-W (400 × g, 5 min) and permeabilized with PBS plus 0.5% of saponin. Subsequently, the cells were washed with PBS-W by centrifugation (400 × g, 5 min) and then incubated with cytokine-specific antibodies against IFN-γ, TNF-α, IL-10 (Miltenyi Biotec, Bergisch Gladbach, Germany) and IL-4 (BD Bioscience, San Jose, CA), all labeled with phycoerythrin (PE), for 30 min, RT. After this time, they were washed by centrifugation with PBS-W (400 × g, 5 min) and resuspended with 1% paraformaldehyde in PBS. The samples were then analyzed (20,000 events/tube) through flow cytometry (FACSCalibur-BD, San Jose, CA) using the software CELL-QuestPro™ (BD Bioscience, San Jose, CA) for acquisition and analysis of data.

2.6. Peripheral blood immunophenotypic analysis

To determine the percentage of CD4^+ and CD8^+ T cells, 5 ml of peripheral blood were collected in tubes containing EDTA. Subsequently 100 µl blood aliquots were transferred to cytometry tubes containing monoclonal antibodies specific for the cell surface markers anti-CD4 or anti-CD8 conjugated with FITC (BD Bioscience, San Jose, CA) and incubated for 30 min. Afterwards, the samples were submitted to erythrocytes lysis, followed by 2 washes and centrifugations (7 min, 300 × g), with posterior resuspension in 400 µl of a 1% paraformaldehyde PBS solution. The cells immunophenotypic and morphometric parameters were determined by flow cytometry (FACSCalibur-BD, San Jose, CA) using the software CELLQuestPro™ (BD Bioscience, San Jose, CA) for acquisition and analysis of data (50,000 events/tube).

2.7. Analysis

The analyses were done first delimitating the lymphocytic region in a Forward Scatter (FSC) versus Side Scatter (SSC) graph. Based on this region the fluorescence graphs (FL1 × FL2) were constructed, delimitating the quadrants for analyses. The quadrant limits were based on the negative population and previous antibodies titration. The values considered for the fluorescence analysis were the percentage of the quadrant region for each quadrant.

The statistical analyses were performed through SPSS 8.0 and GraphPad Prism 5.1, using nonparametric tests. For intragroup comparative analysis, the Kruskal–Wallis test was used and to detect differences between groups, the Mann–Whitney U-test, based on the two-tailed p value. The results were analyzed considering the value of p < 0.05 (statistically significant).

3. Results

3.1. CD4^+ and CD8^+ T lymphocytes percentage in PBMC cultures after stimulation with *Leishmania* soluble and insoluble antigens: distinct patterns

In a phenotypic analysis of responding T cells after culture, we could observe that patients cells before and after treatment presented a superior and significant percentage of CD4^+ T cells when compared to the control group (BT × CT: p = 0.018 SOL/p = 0.040 INS; PT × CT: p = 0.037 SOL/p = 0.022 INS) and also superior when compared to the spontaneously healed group. The percentage of CD4^+ T cells from patients that spontaneously healed was superior to the one of the control group, but without statistical significance (Fig. 2).

The percentage of the CD8^+ T cells in cultures, on the other hand, was inferior to controls in patients before treatment, but increased substantially and significantly after treatment (PT × CT: p = 0.033 SOL/p = 0.025 INS). The percentage of CD8^+ T cells in patients that spontaneously healed was also higher and significantly different when compared to patients before treatment (SH × BT: p = 0.023 SOL/p = 0.021 INS). When the insoluble leishmania antigenic fraction was used the percentage of T CD8^+ cells from SH were also significantly different when compared to controls (SH × CT: p = 0.028 INS) (Fig. 2).

3.2. T CD4^+ and T CD8^+ comparison in ex vivo and culture assays demonstrated a lower CD4/CD8 ratio in PT and SH patients

In order to demonstrate the CD4^+ and CD8^+ T cellular profile during an immunological response, we performed a comparison between the percentage of these lymphocytes in the peripheral blood of patients and controls in an ex vivo context and after PBMC culture with the soluble (SOL) and insoluble (INS) antigenic fractions of *L. (V.) braziliensis*.

Patients showed no significant differences when comparing the percentage of CD4^+ T lymphocytes in the ex vivo assays and in cultures with the antigens. Regarding CD8^+ T lymphocytes it is interesting to note that SH and PT patients, presented higher levels of these cells after stimulation with the SOL and INS antigens. This difference was statistically significant when comparing the percentage of CD8^+ T cells in the ex vivo situation with the CD8^+ T cells after stimulation with the soluble and insoluble antigenic fraction (ex vivo × SOL p = 0.006; ex vivo × INS p = 0.001) (Fig. 2).

Comparing the CD4/CD8 ratio in the ex vivo assays and in the PBMC cultures, we found a variation in the values presented by the groups. Patients BT, PT and SH in the ex vivo assays, as well BT cultures, showed a CD4/CD8 ratio >2, while SH patients in the ex vivo assays and the PT and SH after antigenic stimulation with the antigenic fractions showed a ratio <2 (Table 2).

3.3. Leishmania antigens induced different cytokine profiles, with a predominant Th2 profile in the beginning of infection and a Th1 one after therapy

The cellular source and frequency of cytokine-producing cells induced by antigenic fractions of *L. (V.) braziliensis*, were determined after culture. When the soluble and insoluble antigenic fractions were used, different profiles were observed. After stimulation with the antigens, CD8^+ T cells were the main responsible for the production of IFN-γ and TNF-α, the latter only in BT patients. CD4^+ T cells were mainly producers of IL-10 and IL-4, besides TNF-α in PT and SH patients (Figs. 3 and 4).

The soluble antigen fraction stimulated CD4^+ T to produce IL-10 (BT × CT p < 0.0001) and IL-4 (BT × CT p = 0.030), and CD8^+ T cells
to produce TNF-α, IL-4 and IL-10 (BT × CT p = 0.016) before treatment. After treatment there was a change in this profile, with a decreased in the production of IL-4 and IL-10 (BT × PT p = 0.003) and significant increase in the production of IFN-γ (PT × BT p = 0.023) and TNF-α (PT × BT p = 0.0002/PT × CT p = 0.005) by CD4+ T cells. However, patients still had a significant production of IL-10 (PT × CT p = 0.005) by CD4+ T cells. CD8+ T had a similar profile, with a decrease in IL-4 and IL-10 production (BT × PT p = 0.008), and a significant increase in TNF-α (PT × BT p = 0.014) and IFN-γ (PT × BT p = 0.020) by CD4+ T cells. SH Patients showed a higher production of TNF-α, IFN-γ and IL-4 by CD4+ T cells and TNF-α and IFN-γ (SH × BT, p = 0.046) by CD8+ T cells. When considering the production of IL-10 by SH group, significant difference was seen in the production of CD4+ T cells (BT × SH p = 0.037) SH × CT p = 0.019) (Fig. 3A and B).

The insoluble antigenic fraction stimulated a significant production of IL-10 (BT × CT p = 0.005) and IL-4 (BT × CT p = 0.028) by CD4+ T cells before treatment. The CD8+ T cells produced a relevant amount of IL-10 (p = 0.004) when comparing BT × CT. After treatment, an increase in the production of IFN-γ and TNF-α was seen, being the last one significant between patients PT × BT by CD4+ (p = 0.003) and CD8+ (p = 0.006) T cells. When considering CD8+ T cells the production of IL-10 and IL-4 before and after treatment was similar. Significant difference was seen in the percentage of IL-10 (p = 0.020) between the PT × CT by CD4+ T cells. The SH group presented a higher production of TNF-α (T CD4+, SH × CT p = 0.002) and a lower production of IL-10 when compared to the one of CD4+ (p = 0.008) and CD8+ (p = 0.001) T cells from BT × SH and with CD8+ T cells of PT × SH groups (Fig. 3A and B).

### Table 2

<table>
<thead>
<tr>
<th>CD4+/CD8+ T cell ratio</th>
<th>Ex vivo</th>
<th>Soluble antigen</th>
<th>Insoluble antigen</th>
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<tr>
<td>BT</td>
<td>2.39</td>
<td>2.6</td>
<td>2.38</td>
</tr>
<tr>
<td>PT</td>
<td>2.29</td>
<td>1.4</td>
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<td>1.69</td>
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<tr>
<td>CT</td>
<td>2.48</td>
<td>1.71</td>
<td>1.74</td>
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(to be continued)
were not induced in patients after chemotherapy, as also implied in the study by Botelho et al. [23].

Studies have shown that antigens from the promastigote forms of *L. (V.) braziliensis*, intact or sonicated, were able to induce different levels of cellular immune response [4–6,12,18–20,25,26,31] and noted that the search for antigenic molecules is relevant for the identification of new subunit vaccine candidates and targets for immunotherapy. This reinforces the importance of our study.

Fig. 3. Cytokine expression by (A) CD4+ and (B) CD8+ T cells after *in vitro* stimulation with: *Leishmania* soluble antigenic fraction (1.25 μg/ml) and *Leishmania* insoluble (2.25 μg/ml) antigenic fraction. The results are expressed as mean values ± SEM. The differences were considered significant at *p* less than 0.05 and are represented by the symbols: ◼ = difference between PT × BT; □ = difference between BT × SH; ◊ = difference between SH × CT; ● = difference between PT × CT; □ = difference between PT × SH; ○ = difference between BT × CT; □ = difference between BT × PT. BT = before treatment/PT = after treatment/SH = spontaneously healed/CT = controls.

Fig. 4. Representative FACS dot plots of cytokine expression by (A) CD4+ and (B) CD8+ T cells.
seeking to characterize immunologically the soluble and insoluble fractions of *L. (V.) braziliensis*.

This study, comparing the evolution of patients BT, PT and SH observed a significantly production of IL-10 and IL-4 by CD4⁺ T lymphocytes in BT patients. An inversion of this situation, with increased production of IFN-γ and TNF-α, was seen after treatment with Glucantime®, where IFN-γ was mainly produced by CD8⁺ T cells and TNF-α by CD4⁺ T cells. The SH patients exhibit a predominance of type 1 profile with IFN-γ and TNF-α, however, still with production of Th2 cytokines. The PT patients showed similar results to the ones of SH, suggesting that this is an immunological profile associated with healing. In addition to reinforcing the importance of a type 1 immune response in the evolution for a healing and immunity from the disease, our work also showed that the presence of IL-10, an inhibitory cytokine, is essential as a counterbalance and control of an exacerbated immunopathology, a result also suggested by other groups [11,19].

In studies with *L. (V.) braziliensis* it was found that in early ACL (<60 days) it is observed a preferential production of IL-10 and that there is a low or absent IFN-γ production. This pattern then evolves into a predominantly Th1 cytokine profile, where patients have IFN-γ production and lymphoproliferation [11,27,28]. Our results and those of other groups suggest that susceptible individuals after exposure to the parasite develop a type 2 immune response, allowing parasite survival and growth and the development of lesions. With the progression of the disease, this response changes to type 1 profile, activating macrophages, which leads to the control of parasite growth and disease progression. Therefore, it is possible that the development of the disease depends on a temporary immune dysregulation during the early stages of ACL [11,11], since works correlate the participation of IL-10 and IL-4 with the evolution of ACL [27,29,30].

Most patients with ACL heal after treatment with antimonials, which indicates a later development of an appropriate and beneficial immune response [7,27,28]. In the present study we saw that there was a significant increase in production of TNF-α and IFN-γ in PT patients. It is interesting to note that SH patients had the same profile, with the production of TNF-α and IFN-γ, even in the absence of chemotherapy. In endemic *L. (V.) braziliensis* areas spontaneously cured patients have been documented, showing this immune response profile even without treatment [7,24]. This result reinforces the importance of the type 1 response, and demonstrates that it is necessary to control the multiplication and dissemination of *Leishmania*. Although there is no clinical or laboratory test to predict which patients will respond or fail to treatment with antimonials, it is likely that patients producing high levels of TNF-α and IFN-γ have a better prognosis for cure in infections with *L. (V.) braziliensis*.

Although, however a type 1 immune response is critical for the control of leishmaniasis, the loss of an appropriate modulation is responsible for the immunopathology [28]. In our study IL-10 production by CD4⁺ T lymphocytes in PT patients when stimulated with both antigenic fractions and by SH patients when stimulated with the soluble antigenic fraction, was not associated with a low production of IFN-γ and TNF-α. This indicated a coordinated immunoregulation of T cells that produce protective and potentially pathogenic cytokines, a situation that leads to the resolution of the ACL infection. Therefore, as evidenced by our PT and SH patients, a proper balance between the responses that induce a leishmanicidal activity, those that induce the disease, and those that maintain the parasite persistence may be the most desired type of response against infection by *L. (V.) braziliensis*.

The immune pattern observed in the current study appears to be associated with the healing and/or protection in ACL, suggesting the existence of memory T cells and immunoregulatory mechanisms, whose function were triggered by our antigens. Patients’ treatment can be benefited by immunological interventions if the exact role of T cells subtypes in the disease and resistance can be clarified [7,12,17,29]. The immune response developed by patients against the antigens will be useful in determining an active disease prognosis and the necessary mechanisms for its evolution to cure. The observed result may also be an important parameter for future selection of antigens candidates for vaccine and prognostic tests, since the development and maintenance of a protective immune profile is very important and there is not yet a well defined antigen for the quest. This investigation adds informations to the search of factors that contribute to the susceptibility and putative protective immunity against human cutaneous leishmaniasis in *L. (V.) braziliensis* infection and provides additional information on the search for relevant antigens in this disease.

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**References**


