

Protective profile involving CD23/IgE-mediated NO release is a hallmark of cutaneous leishmaniasis patients from the Xakriabá indigenous community in Minas Gerais, Brazil

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ABSTRACT

In the present study we described, for the first time, specific aspects of an anti-*Leishmania* immune response in a Brazilian Xakriabá indigenous community. Induction of an intracellular NO pathway, triggered by the binding of IgE to CD23 receptor in IFN- γ /IL-4 cytokines environment, were evaluated in Localized Cutaneous Leishmaniasis (LCL) carriers and positive Montenegro Skin Test (MST) individuals without skin lesion (MT⁺SL⁻). Our data demonstrated that the higher frequency of CD23⁺CD14⁺ monocytes and the increased serum levels of IgE observed in the LCL group were significantly higher in LCL carriers with late lesions (LCL $\geq$ 60). Furthermore, LCL patients presented increased NO production after *Leishmania (Viannia) braziliensis* stimulation and this NO profile was independent of the age of the lesion (recent-LCL<60 or late-LCL $\geq$ 60). We also showed that the increased frequency of IFN- γ ⁺ and IL-4⁺CD4⁺ T-cells are related with the MT⁺SL⁻ group. The results of the evaluation of biomarker signature curves demonstrated that in the MT⁺SL⁻ group the index signature was characterized by DAF-2T⁺CD14⁺/ IL-4⁺CD8⁺/ IFN- γ ⁺CD4⁺/ IL-4⁺CD4⁺. On the other hand, the LCL group presented a higher index of DAF-2T⁺CD14⁺/ CD23⁺CD14⁺/ IL-4⁺ CD8⁺, associated with a lower index of IFN- γ ⁺CD8⁺. Considering lesion time, analysis of the LCL group demonstrated that the main differences observed were highlighted in LCL<60 patients, with a higher index of CD23⁺CD14⁺, which was also present in LCL $\geq$ 60 patients. In conclusion, our data suggest that the protective immune response involving CD23-IgE-mediated NO release is a hallmark of LCL patients. However, in MT⁺SL⁻ individuals another different leishmanicidal mechanism seems to be involved.

INTRODUCTION

In the New World, leishmaniasis is a zoonosis that involves different species of protozoans of the genus *Leishmania* and a great variety of mammal hosts and reservoirs. In human leishmaniasis, the clinical manifestations depend mainly on the species of the parasite involved and the immune response of the infected host, however, it is important to emphasize that many factors together function to determine the complex spectrum of clinical manifestations of human leishmaniasis. Tegumentary Leishmaniasis (TL) has a great variety of clinical manifestations with the most common being Cutaneous Leishmaniasis (CL). In CL innate immunity is preserved, which can be observed through the positive Montenegro skin test result (MST). This specific cellular response, which is well-modulated but with a predominance of type 1 cytokines, reflects the tendency of spontaneous healing and positive response to treatment in patients with CL. Furthermore, about 10% of individuals who live in endemic areas present positive MST results yet do not develop the disease, presenting instead a subclinical form of TL [1].

There has been little research on the occurrence of TL outbreaks in indigenous tribes in Brazil. The earliest of these took place in the 1950's and 1960's and studied tribes located in the state of Mato Grosso [2,3]. Aspects of the lifestyle and social condition of indigenous populations, such as poor nutrition, lack of hygiene and sanitation, close interaction with animals in the domestic and peridomestic environments, all favor the transmission of many parasitoses [4], including TL. In a Xakriabá indigenous community, for example, studies revealed that *L. (V.) braziliensis*, *L. (L.) infantum* and *L. (V.) guyanensis* are circulating among domestic and synanthropic mammals, thus favoring leishmaniasis transmission in this area [5]. Additionally, a study related to immune response was undertaken in the same community and was able to characterize the immunological biomarkers of the skin lesions of these Brazilian Indians [6].

Some previous research has been attracting attention for its importance regarding patterns of immune response in TL. Many studies suggest that the type 1 cellular immune response, characterized by the production of IFN- γ , serves a protective role [7,8]. In murine models, IFN- γ has shown a simultaneous action with another cytokine, the TNF- α , in activating inducible nitric oxide synthase (iNOS or NOS2) to produce nitric oxide (NO) resulting in the killing of intracellular parasites [9,10]. Vouldoukis et al. (1995) demonstrated that the binding of IgE to the low affinity receptor Fc ϵ RII /CD23 in human macrophages is strong, and induces NO production after activation with IL-4 and IFN- γ or IFN- γ only, thus restricting the growth of *Leishmania major* [11]. Moreover, it has been described that serum IgE levels and CD23 expression are increased in CL, and human monocytes/macrophages express Fc ϵ RII/CD23 after activation with IFN- γ or IL-4 [12,13].

In this context, the present study evaluated, for the first time, specific aspects of an anti-*Leishmania* immune response in a Brazilian indigenous community. Induction of the intracellular NO pathway, triggered by the binding of IgE to the CD23 receptor in IFN- γ /IL-4 cytokines environment in CL carriers and positive MST individuals without skin lesions were evaluated in the Xakriabá indigenous community in the state of Minas Gerais, Brazil. This study provided a rare opportunity to study immunological aspects related to CL in an indigenous population.

POPULATION, MATERIAL AND METHODS

Study area

The Xakriabá Indigenous Reserve is located in the municipality of São João das Missões (14°53'01" S, 44°05'26" W), in northern Minas Gerais, Brazil. The reserve covers 78% of the entire municipality and has a population of 7,813 inhabitants. Between 2001 and

2012, 402 cases of cutaneous leishmaniasis (CL) were recorded in the Xakriabá territory, and since 2008 a large study has been done in the reserve to address measures to prevent and control the transmission of CL. All actions have been conducted by the Family Health Program, currently adopted by the Brazilian Unified Health System, and also located in the Xakriabá Territory.

Study population

The study included indigenous patients with a diagnosis of localized cutaneous leishmaniasis (LCL, n=17) during the 4-year period of 2009-2012 (Table 1). The selection criteria for inclusion in the study were patients with skin lesions suggestive of LCL who had a positive Montenegro skin test (MST), presence of *Leishmania* parasites documented by Giemsa-stained imprints of biopsy fragments and/or parasite isolation in culture medium containing blood agar enriched with LIT (Liver Infusion Tryptose) medium maintained at 26°C. Furthermore, DNA detection of *Leishmania* by PCR (*kDNA* was used as DNA target) also made patients eligible for inclusion. Any patients with alcohol dependence, tuberculosis, diabetes, pneumonia HIV infection or other immunosuppressive disease, use of immunosuppressive agents, pregnancy, patients with severe anemia, and patients with previous specific leishmaniasis treatment were excluded. All patients received specific treatment for LCL at the Programa de Saúde da Família (Family Health Program) as recommended by the Brazilian Ministry of Health guidelines.

In this study, it were included individuals with positive Montenegro skin test without skin lesions (MT⁺SL⁻, n=13), and non-infected individuals who did not present suggestive signals of active LCL or a characteristic scar, plus negative MST (NI, n=10), all residents in Xakriabá Indigenous Community (Table 1). For future comparative analysis, the LCL group was divided in two other groups according to the duration of lesion; patients with early

lesions and patients with late lesions. Patients with early lesions were, those whose lesion presented a development time of less than 60 days ($LCL < 60$, $n = 7$), whereas patients with late lesions were those whose lesions presented development time of greater than or equal to 60 days ($LCL \geq 60$, $n=10$).

Ethical issues

The study was conducted in agreement with the Helsinki Declaration, Resolution #466/2012 at the Comissão Nacional de Ética em Pesquisa – CONEP – (National Commission on Research Ethics) that regulates research involving human subjects in Brazil, and Resolution #304/2000 that regulates research involving Indians. The Comitê de Ética em Pesquisa (Research Ethics Committees) at Centro de Pesquisas René Rachou (CEPSH/CPqRR n°: 021/2007), and at the CONEP (protocol n°.14689) previously approved the informed consent forms and procedures. Written informed consent was obtained from all adults, and the children were formally included in the study only if the informed consent form was signed by their parents or legal guardians. This project was conducted with the permission of the Fundação Nacional do Índio – FUNAI – (Indigenous National Foundation, license n°. 149/CGEP/08) to enter into indigenous lands.

L. (V.) braziliensis FITC- labeling procedures

Promastigotes of *Leishmania (V.) braziliensis* (MHOM/BR/1986/MSS) were obtained from cultures in complex liquid medium LIT supplemented with 20% fetal bovine sera (FBS) maintained at 26°C in a B.O.D. incubator for 7 days [14,15]. The parasites were stained with fluorescein isothiocyanate (Fluorescein Isothiocyanate, Isomer I - $C_{21}H_{11}NO_5S$ - FITC; Sigma, MO, USA) at a final concentration of 200 $\mu\text{g/mL}$ at 37°C for 30 min in a 5% CO_2 humidified incubator. After the FITC-labeling procedure, parasites were washed three times with PBS and resuspended in PBS supplemented with 10% FBS. The FITC-labeled parasites

suspension was adjusted to 1×10^8 /mL, and maintained at 26°C in a B.O.D. incubator until use. Aliquots of FITC-labeled parasites were run in a flow cytometer to evaluate the efficiency of the FITC staining procedure. The ideal FITC-labeled parasite staining would lead to a single peak around 10^2 and 10^3 log intervals in FL1/FITC histogram plots. Additionally, the FITC-labeled parasites were monitored by optical microscopy for motility features and by trypan blue staining for viability quality control.

Phagocytosis assay of L. (V.) braziliensis promastigotes by peripheral blood monocytes

Heparinized peripheral blood was centrifuged at $1200 \times g$ for 10 min at room temperature. The plasma was removed and the blood cells were washed with PBS supplemented with 10% FBS, and the final cell suspension was adjusted to 1×10^7 cells/mL. Short-term *in vitro* cultures were performed in two distinct platforms referred to as “Ex vivo – Unstimulated” and “*L. braziliensis*-Stimulated” cultures. For the “Ex vivo – Unstimulated” cultures aliquots of 1 mL of leukocyte suspension were incubated with 1 mL of PBS supplemented with 10% FBS. For the “*L. (V.) braziliensis* – Stimulated” cultures, aliquots of 1 mL of leukocyte suspension were incubated with 950 μ L of PBS supplemented with 10% FBS and 50 μ L of FITC-labeled live *L. (V.) braziliensis* organisms at 1×10^8 parasites/mL. Both, unstimulated and stimulated cultures were performed in duplicate. The tubes were incubated under gentle shaking by an orbital shaker for 120 min at 37°C in a 5% CO₂ humidified incubator. After incubation, each tube received Brefeldin A - BFA (Sigma, MO, USA) at 10 μ g/mL and re-incubated for 240 min at 37°C in a 5% CO₂ humidified incubator. Following the incubation, the cultures were treated with EDTA at 20 mM, and maintained at room temperature for 15 min prior to immunophenotypic staining for cell surface markers and intracytoplasmic cytokine analysis.

Analysis of cell surface marker and intracytoplasmic cytokines

For cell surface marker analysis aliquots of 200 μ L cell cultures obtained from the phagocytosis assay were incubated for 30 min at room temperature with PerCP-labeled anti-CD14 mAb and phycoerythrin (PE)-labeled anti-CD23 mAb, all purchased from Becton Dickinson. Following incubation, the red blood cells were lysed with FACS lysing solution, washed with FACS buffer, and fixed with FACS FIX solution. A total of 3,000 monocytes were obtained and analyses of the immunophenotypic and morphometric parameters were determined in a FACScan flow cytometer.

For intracytoplasmic cytokine analysis, aliquots of 500 μ L cell cultures were incubated for 30 min at room temperature with anti-CD4PerCP and anti-CD8PerCP mAbs to identify T Lymphocyte subsets. Following incubation, the red blood cells were lysed with FACS lysing solution and the samples were kept with FACS permeabilizing solution. Following incubation, the samples were centrifuged at 600 x g for 7 min at room temperature and the cell pellet washed with FACS buffer. After centrifugation, the cells were resuspended with FACS buffer. Cells were then stained with anti-cytokine MoAbs, including anti-IFN- γ PE and anti-IL4PE, by incubation for 30 min at room temperature in the dark. After incubation, the cells were washed twice and fixed with FACS FIX solution. A total of 20,000 Lymphocytes were acquired and analyzed. Morphometric, phenotypic and functional parameters were determined in a FACScan flow cytometer.

Intracellular nitric oxide (NO) levels

In the present study, the intracellular NO assay was performed using plasma-free whole blood samples as previously reported by Schachnik et al (2009) [16] and Gomes et al (2012) [17]. Plasma-free whole blood was resuspended to 1×10^7 leukocytes/mL in PBS 2% BSA. Fifty microliters of leukocytes were pre-incubated with *L. (V.) braziliensis* soluble

("LSA" culture) antigen preparation at a final concentration of 25µg/mL at 37°C for 120 min in a 5% CO₂ humidified incubator. Control cultures ("Ex vivo – Unstimulated" cultures) were used to determine the basal levels of intracellular nitric oxide. The samples were then incubated in the presence of 4,5- diaminofluorescein-diacetate (DAF-2DA) (Sigma, MO, USA) (final concentrations of 2.0 µM) at 37°C for 180 min in a 5% CO₂ humidified incubator. The samples were labeled and placed in an ice bath for 20 min in the presence of PerCP-labeled anti-CD14 mAbs. The cells were lysed with FACS lysing solution, washed with PBS solution and the cell pellet was gently resuspended with PBS solution for immediate flow cytometer acquisition in a FACScan flow cytometer. A total of 50,000 events/tube were acquired. Positive controls were performed by pre-incubation of leukocyte samples for 60 min with a NO inducer (LPS 10 µg/mL, Sigma, USA) or 10 min pre-incubation with NO inhibitors (N ω -nitro-L-arginine methyl ester 10 mM, and Aminoguanidine (10 mM, Sigma, MO, USA) at 37°C in a 5% CO₂ humidified incubator (data not shown). The data acquired were analyzed using FlowJo software.

Flow cytometric analysis

Distinct gating strategies were used to analyze CD23 expression, cytokine profile and NO production of specific leucocyte subpopulations. A strategic gate was created for selective analysis of monocytes, gated as SSC^{high}CD14^{high} cells, using FL3/anti- CD14-PerCP versus laser side-scatter (SSC) graph (Figure 1A and 1D). Following the gating procedure, the percentage of monocytes CD14⁺CD23⁺ and NO-producers was determined using double positive quadrant of FL2/anti-CD23-PE versus FL3/anti-CD14-PerCP and FL1/DAF-2T versus FL3/anti-CD14-PerCP dot plots, respectively (Figure 1B, 1C and 1E). Lymphocyte scatter gate was set-up using FSC versus SSC dot plots (Figure 1F). Cytokine-expressing T

CD4 and T CD8 lymphocyte cells were identified using FL3/anti-CD4 and anti-CD8-PerCP versus FL2/anti-IL-4 and anti-IFN- γ -PE dot plots (Figure 1G).

Biomarker Signature Analysis

Biomarker signatures were assembled as previously reported by Luiza-Silva et al. (2011)[18]. The global median value for each biomarker (CD23, NO, IL-4 and IFN- γ) was calculated using all the groups values (LCL, LCL<60, LCL $\geq$ 60, MT⁺SL⁻ and NI). The global median biomarker index (LEISH-LSA/CC) was used to tag each individual as they display “Low” or “High” biomarkers levels. This strategy allowed for a final computation of the percentage of individuals displaying “High” biomarker index. Next, the “ascendant biomarker signature” for the NI group was assembled and taken as the “reference curve” to highlight the changes in biomarker profiles of TM⁺SL⁻, LCL, LCL<60 and LCL $\geq$ 60 groups. Additionally, the “ascendant biomarker index signature” for each clinical group was also assembled and overlaid to identify changes in the overall biomarker profile.

Data analyzes

The identification of the cell populations of interest, determination of the phagocytic capacity, and percentage of phenotypic markers were evaluated though FlowJo software (Flow Cytometry Analysis Software, version 7.6.1.). Statistical analyses were performed using GraphPad Prism software (San Diego, USA, version 5.00.) For comparative analyses between two groups the Mann Whitney test was used, whereas for comparative analyses among three groups, the Kruskal-Wallis test was used followed by Dunn’s post-test. In all cases, differences were considered statistically significant when the *p* value was less than 0.05.

Total IgE concentration

A specific CAP-fluorescein-enzyme immunoassay (Cap-FEIA), ImmunoCAP Total IgE kit - Thermo Scientific™, was used to quantify the serum level of total IgE as per the manufacturer's instructions. For serum measurements, the samples were stored at -70°C until use. The results were considered elevated when total IgE concentration was higher than 116 KU/L for individuals of 7-10 years old and higher than 140 KU/L for individuals >10 years old [19].

RESULTS

The higher frequency of monocytes expressing CD23 and NO after Leishmania braziliensis stimulation and increased serum levels of IgE are a hallmark of the LCL group

Here we evaluated the impact of *L. (V.) braziliensis* on CD23 and NO expression by peripheral blood monocytes and serum levels of IgE in MT⁺SL⁻ individuals and LCL patients. Our data demonstrated that *L. (V.) braziliensis* stimulation (LEISH or LSA) induced significant increase in the CD23 (MT⁺SL⁻ – $p=0.0488$; LCL – $p=0.0008$; LCL<60 – $p=0.0043$; LCL≥60 – $p=0.0107$) and NO (NI – $p=0.0093$; MT⁺SL⁻ – $p=0.0001$; LCL – $p=0.0001$; LCL<60 – $p=0.0047$; LCL≥60 – $p=0.0002$) expression by monocytes in all groups as compared with the control culture (CC) (Figure 2A and 2B).

Data analysis demonstrated that the LCL group presented a high frequency of CD23⁺CD14⁺ monocytes when compared with NI ($p=0.0079$) and MT⁺SL⁻ ($p=0.0070$), and that this increase was significantly higher in LCL≥60 ($p=0.0118$; $p=0.0121$, respectively) (Figure 2A).

Aiming to quantify NO production, we performed an analysis of the percentage of DAF-2T⁺CD14⁺ monocytes in peripheral blood. The results showed that independent of the time lesion (LCL<60 or LCL≥60) the LCL group presented a high frequency of NO producing monocytes as compared to the NI ($p=0.0155$) and MT⁺SL⁻ ($p=0.0273$) groups (Figure 2B).

Interestingly, we observed elevated IgE serum levels in in patients with late LCL lesion (LCL≥60) as compared with the MT⁺SL⁻ ($p=0.0492$) and LCL<60 ($p=0.0423$) groups (Figure 2C).

Increased frequency of IFN- γ ⁺ and IL-4⁺ CD4⁺ T-cells are related with MT⁺SL⁻ group

Aiming to evaluate an important NO induction pathway we characterized the cytokine profile by circulating T Lymphocytes in the absence/control culture (CC) and in the presence of *L. braziliensis* (LEISH) stimuli. For this purpose we analyzed the intracytoplasmic expression of IFN- γ and IL-4 by CD4⁺ and CD8⁺ T-cell subsets from the NI, MT⁺SL⁻ and LCL groups (Figure 3). Data analysis demonstrated that the increased frequency of IFN- γ ⁺ and IL-4⁺ CD4⁺ T-cells (Figure 3A and 3C, respectively) are related with MT⁺SL⁻ group when compared with NI (IFN- γ → $p=0.0007$; IL-4 → $p=0.0170$), LCL (IFN- γ → $p=0.0184$; IL-4 → $p=0.0129$), LCL<60 (IFN- γ → $p=0.0216$; IL-4 → $p=0.0075$) and LCL≥60 (IFN- γ → $p=0.0343$; IL-4 → $p=0.0327$), despite the absence of significant alterations of CD8⁺ T-cells in the evaluated groups (Figure 3B and 3D).

High producers from MT⁺SL⁻ individuals display a unique cytokine profile by CD4⁺ T-cells and L. (V.) braziliensis stimuli induced significant impact in the CD23 and NO expression by monocytes in LCL patients

In a different analysis, the index of the high producers of each group was evaluated for each cellular profile tested. The high producers in the NI group were then aligned to create a reference biomarker index signature that was later on ordered in an ascendant fashion (Figure 4A). The high producers in the other groups were also aligned following the same sequence observed in the NI group for clearer comparisons and overlaid with the reference curve created for the NI group. The results demonstrated that the profile observed in the MT⁺SL⁻ group is well above the NI reference biomarker index signature, especially for DAF-2T⁺CD14⁺, IL-4⁺CD8⁺, IFN- γ ⁺CD4⁺ and IL-4⁺CD4⁺ (Figure 4B). On the other hand, when evaluated in the LCL patients, it was possible to see that the high producers presented a higher index of DAF-2T⁺CD14⁺, CD23⁺CD14⁺ and IL-4⁺CD8⁺, associated with a lower index of IFN- γ ⁺CD8⁺ high producers (Figure 4C). Considering lesion age, analysis of LCL demonstrated that the main differences observed in the LCL group were that LCL<60 patients had a higher index of CD23⁺CD14⁺, which was also present in LCL $\geq$ 60 patients (Figure 4D and 4E, respectively).

DISCUSSION

It has been shown that the different clinical forms of human cutaneous leishmaniasis present distinct immunological features. During the development of the anti-*Leishmania* immune response the secretion of soluble factors occurs, such as cytokines and chemokines, associated with the recruitment of circulating monocytes, which differentiate into macrophages in the inflammatory process [20,21]. Macrophages are important cells in leishmaniasis because these cells are targeted by the parasites, thus enabling its development [22,23]. The role played by macrophages depends on the kind of activation and the vulnerability of the parasite against the effector mechanism [24]. The intracellular survival of

Leishmania is related to its ability to prevent the activation of macrophages [25]. Once activated, these cells are able to destroy the parasites, mainly via production of NO and reactive intermediate oxygen (RIOs) [24]. NO is involved in many physiological and pathological processes. In this context, its biological importance is due to the fact that this molecule has the ability to activate or inhibit target molecules in different processes such as regulation of vascular tone, neurotransmission and immune response [26,27]. Guzik et al. demonstrated that during the development of the inflammatory process the synthesis of large amounts of NO via iNOS occurs (inducible nitric oxide synthase enzyme which catalyzes the formation of NO) [28]. This NO, produced mainly by activated macrophages, has cytostatic and cytotoxic properties thus promoting the destruction of microorganisms and tumor cells [28]. Diefenbach et al. (1998) showed a dual function of NO. First, during the innate phase of infection, involving the control of NK cell activity, the early cytokine response and parasite spreading, while during the late phase NOS2 is further up-regulated by IFN- γ -producing CD4+ T cells, and its antimicrobial activity is critical for the reduction of parasite burden and clinical resolution of the disease [29]. It has been demonstrated that topical treatment of *L. major*/*L. braziliensis* ulcers with donor NO was very effective at reducing the size and healing the skin of lesions in mice. These authors suggest that topical application of an NO donor molecule to the lesion may partially compensate for the shortage of NO and promote healing. In addition, the topical therapy of cutaneous leishmaniasis in the mouse model provides the experimental basis for a possible trial of NO in the treatment of LCL [30]. Thus, NO is very important since it is associated with the destruction of parasites and the consequent control of infection.

In the present study we evaluated, for the first time, specific aspects of an anti-*Leishmania* immune response in a Brazilian indigenous community. This was done by investigating aspects of the intracellular NO induction pathway, and its triggering by the

binding of IgE to CD23 receptor in a IFN- γ /IL-4 cytokines environment in CL carriers (LCL) and positive MST individuals without skin lesion (MT⁺SL⁻) of the Xakriabá indigenous community in Minas Gerais, Brazil. We also examined LCL carriers by the age of lesion (recent-LCL<60 and late-LCL $\geq$ 60 days) as a strategy to investigate early and late NO related immunological events that may be triggered by *Leishmania* infection.

Many studies have demonstrated that treatment of human monocytes / macrophages with IFN- γ and IL-4, both secreted during the early phases of the immune response, increases the expression of CD23, the low affinity receptor for IgE [11,31,32]. The binding of CD23 by its ligand increases mRNA transcription of iNOS (inducible nitric oxide synthase), as well as the production of NO [32]. Studies with *Leishmania major* showed that after treatment of human monocytes / macrophages with IFN and IL-4, the infection had not become established, and when the parasites entered the cell they were immediately destroyed by NO, suggesting a protective role of this mechanism [11,33]. Our data, corroborated by these studies, suggests that the protective immune response involving CD23-IgE-mediated NO release is a hallmark of LCL patients, despite the lower percentage of T-cell producers IFN- γ and IL-4. We suggest that other leukocytes such as neutrophils and NK cells may be involved in the expression of cytokines needed to produce NO.

It is important to mention that Gross et al. (2014) affirm that human monocytes do not produce NO under regular cell culture conditions [34]. However, the evaluation of intracellular NO production by monocytes/macrophages has been studied in Leishmaniasis since 2011 [35]. Besides, another study by our group evaluated intracellular NO production by peripheral blood monocytes in cultures in the presence of *Trypanosoma cruzi* trypomastigotes [17].

In general, in LCL the presence of a strong cellular immune response characterized by a positive Montenegro test has been considered a good prognosis [36]. Indeed, it is well

known that in areas endemic for *Leishmania* there are groups of individuals that, although living in these areas, do not develop the disease but yet have positive Montenegro skin test [37]. As mentioned previously, positive MST individuals without skin lesion (MT⁺SL⁻) of the Xakriabá indigenous community in Minas Gerais, Brazil, were evaluated in this study. We identified that the increased frequency of IFN- γ ⁺ and IL-4⁺ CD4⁺ T-cells are related with the MT⁺SL⁻ group. These results were reinforced by a positive correlation between IFN- γ ⁺ and IL-4⁺ cytokines (data not shown). In addition, the analysis of high producers demonstrated that in the TM⁺SL⁻ group the index signature was characterized by DAF-2T⁺CD14⁺/ IL-4⁺CD8⁺/IFN- γ ⁺ CD4⁺/ IL-4⁺CD4⁺. Thus, we suggest that in TM⁺SL⁻ individuals, another NO-dependent leishmanicidal mechanism is involved. Baratta-Masini and collaborators (2007) demonstrated that TM⁺SL⁻ individuals displayed a basal mixed Type-0 cytokines profile. However, they observed a low frequency of IL-4⁺ T-cells, a high IFN- γ ⁺/IL-10⁺ cell ratio, and elevated nitrite and nitrate plasma levels in this group, suggesting that despite basal levels of cytokines, a high proportion of Type-1 over Type-2 cytokines serves to prevent parasite growth and lesion development [29].

It is important to emphasize that there may be immunological differences between indigenous and non-indigenous populations that were not evaluated in the present study. Our hypothesis is that any such differences are likely related to differences in genetic background, behavior, physiology, life history, stress levels, and frequency of contact with *Leishmania* parasites. Another interesting matter that should be discussed is about the parasite contribution to variable clinical manifestations of cutaneous leishmaniasis. A remarkable genetic variability has previously been shown to the *L. (Viannia)* subgenus [38]. Few reports have shown an association between genetic variability in *Leishmania* and variable clinical manifestations. The wide variation in the clinical presentation of cutaneous leishmaniasis with respect to the type, number, shape, size, location and immune outspread of the lesions

can be explained by individual variations in parasite virulence and host susceptibility [39]. Schrieffer *et al.* (2009) studied the genetic variability of *L. (V.) braziliensis* isolates from patients with cutaneous and disseminated leishmaniasis to test the hypothesis that the polymorphism of *L. (V.) braziliensis* could explain these different clinical manifestations [40]. They observed an association between distinct *L. (V.) braziliensis* genotypes and the different clinical forms (cutaneous and disseminated). Results of studies carried out by an our collaborative research group showing that *L. (V.) braziliensis* variants are associated with different lesion types (Quaresma et al., 2015 - *personal communication*) emphasizes the importance of further investigations on the relationship between clinical manifestations and genetic diversity in *L. (V.) braziliensis*. Future studies will be very important in addressing these questions.

CONCLUSIONS

Together, the data presented in this paper demonstrate that there are significant differences in the immune response of the individuals evaluated in this study and that these differences are related to the development of resistance or susceptibility to infection. We suggest that the protective immune response involving CD23-IgE-mediated NO release is a hallmark of LCL patients. However, in TM^+SL^- individuals, another leishmanicidal mechanism seems to be involved. In addition, this study provided a rare opportunity to study immunological aspects related to CL in an indigenous population.

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Figure Legends

Figure 1 Analyses of peripheral blood leucocytes of evaluated population by flow cytometry.

(A) represents the cell profile of monocytes population, selected on a gate in charts of granularity density (SSC) versus fluorescence-3 (anti-CD14-PerCP). (B) represents the monocytes profile in absence of DAF-2DA probe in charts of fluorescence-1 density versus anti-CD14-PerCP. (C) represents the profile of NO-producers monocytes (DAF-2T+) in double positive quadrant (Q2) in charts of fluorescence-1 density (DAF-2T) versus anti-CD14- PerCP. (D) represents the cell profile of monocytes population, selected on a gate in charts of granularity density (SSC) versus fluorescence- (anti-CD23-PE). (E) represents the profile of CD23-expressing monocytes (anti-CD23-PE) in double positive quadrant (Q2) in charts of fluorescence-3 density (anti-CD14-PerCP) versus fluorescence-1 anti-CD23- PE. (F) represents the cell profile of T lymphocytes population, selected on a gate in charts of

granulosity density (SSC) versus cell size (FSC). (G) represents the profile of IL-4-producers T CD4⁺ lymphocytes (anti-CD4-PerCP) in double positive quadrant (Q2) in charts of fluorescence-1 density (anti-CD4-PE) versus fluorescence-3 anti-IL-4-PE.

Figure 2 Column chart of functional monocyte profiles and serum IgE concentrations in healthy controls (NI), individuals with positive Montenegro skin test (MT⁺SL⁻), and patients with Localized Cutaneous Leishmaniasis (LCL) categorized as recent lesion (LCL<60) and late lesion (LCL≥60). The column in gray show median values of percentile and interquartile range and highlight the frequency of monocytes CD23⁺ CD14⁺ (A) and DAF-2T⁺ CD14⁺ (B) for the control culture (CC) and the *Leishmania braziliensis* culture (LEISH or LSA) by flow cytometry and IgE concentration by specific CAP-fluorescein-enzyme immunoassay (Cap-FEIA) (C). Significant differences (P < 0.05) among the groups NI, MT⁺SL⁻, LCL, LCL<60 and LCL≥60 are represented by the letters *a, b, d*. Significant differences between the control culture (CC) and the *Leishmania braziliensis* culture (LEISH or LSA), are represented by asterisks (*).

Figure 3 Column chart of functional T lymphocyte profiles in healthy controls (NI), individuals with a positive Montenegro skin test (MT⁺SL⁻), and patients with Localized Cutaneous Leishmaniasis (LCL) categorized as recent lesion (LCL<60) and late lesion (LCL≥60). The columns in gray show median values of percentile and interquartile range and highlight the frequency of T lymphocyte subsets IFN-γ⁺CD4⁺/IFN-γ⁺CD8⁺ (A and B) and CD4⁺IL-4⁺/IL-4⁺CD8⁺ (C and D) for the control culture (CC) and the *Leishmania braziliensis* culture (LEISH). Significant differences (P < 0.05) among the groups NI, MT⁺SL⁻, LCL, LCL<60 and LCL≥60 are represented by the letters *a, c, d, e*. Significant

differences between the control culture (CC) and the *Leishmania braziliensis* culture (LEISH), are represented by asterisks (*).

Figure 4 Signature profiles of biomarkers for individuals with positive Montenegro skin tests (MT⁺SL⁻), patients with Localized Cutaneous Leishmaniasis (LCL), and healthy controls (NI). LCL patients were categorized as recent lesion (LCL<60) and late lesion (LCL≥60). The graphics were plotted using the global median biomarker index (LEISH-LSA/CC) as the cut-off for identifying each subject as “low” or “high” biomarker producers. (B, C, D) Bar charts showing the frequency of individuals with the “High” biomarker index of DAF-2T⁺CD14⁺, CD23⁺CD14⁺, IL-4⁺CD8⁺, IFN-γ⁺CD4⁺, IL-4⁺CD4⁺ and IFN-γ⁺CD8⁺ in MT⁺SL⁻, LCL, LCL<60 and LCL≥60 groups according to the (A) NI ascendant biomarker signature, used as a “reference curve” (—●—). Relevant changes were considered when the frequency of individuals with “High” biomarker levels shifted towards a distinct 50th percentile as compared to the NI reference curve and are highlighted by arrows (↓↑).

Table 1: Characterization of the study population .

GROUP	AR	GENDER	NL	TL
LCL (n=17)	7-45	10M – 7F	1-9	1-24
MT ⁺ SL ⁻ (n=13)	13-63	6M-7F	-	-
NI (n=10)	14-52	3M-7F	-	-

AG: Age Range (years); NL: Number of lesions; TL: Time of lesions (months).

Analyses of peripheral blood leucocytes of evaluated population by flow cytometry

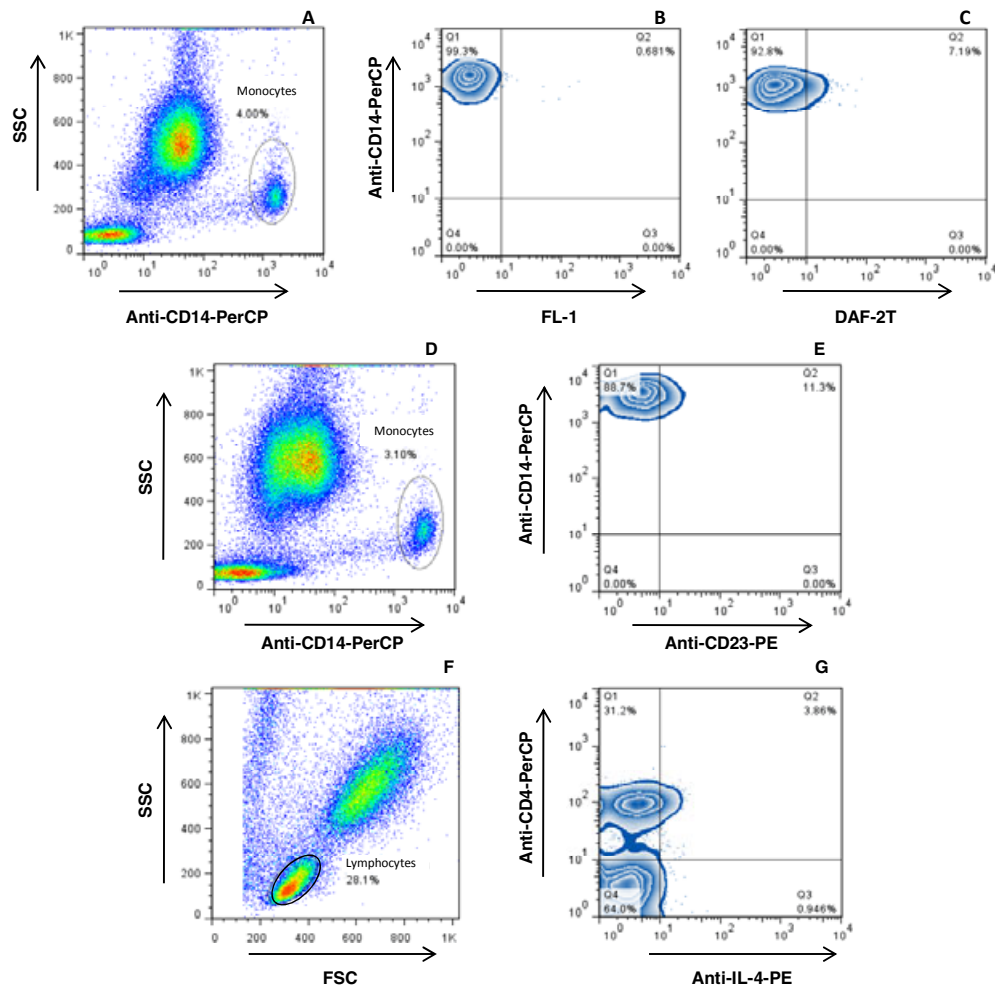


Figure 1

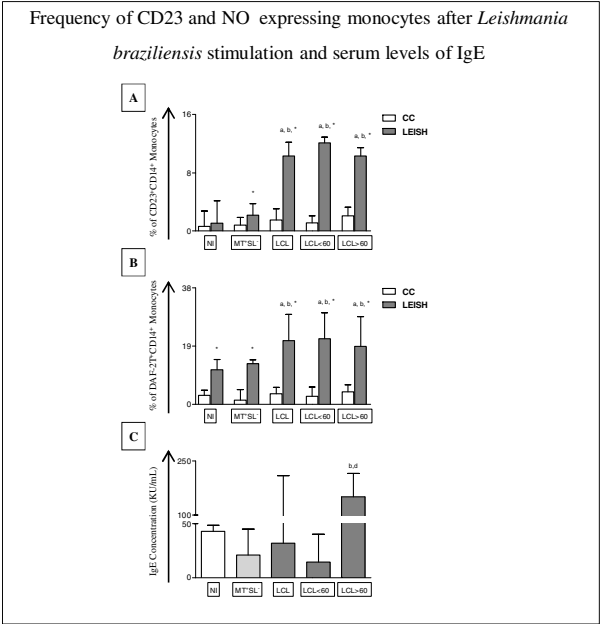


Figure 2

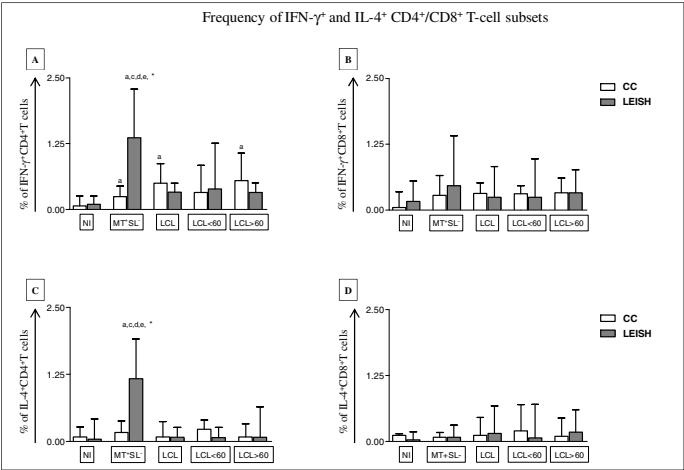


Figure 3

Figure 4

