Mixed cytokine profile during active cutaneous leishmaniasis and in natural resistance

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1. ABSTRACT

Most studies on immune response in human cutaneous leishmaniasis evaluate patients with active disease in comparison with healthy uninfected controls or patients that have had the lesions healed, however, little is known about the immune response associated with natural resistance. In this paper we evaluate the cytokine expression patterns of T-cells and the plasmatic levels of nitrite and nitrate in patients with localized cutaneous leishmaniasis (LCL) as well as endemic non-infected individuals with positive (MST) and negative (NI) Montenegro skin test, without previous history of leishmanial lesions. Our results demonstrated an increased number of IFN-gamma$^+$ and TNF-alpha$^+$ T-cells and high level of plasma nitrite and nitrate in LCL patients. Moreover, we have observed that early in infection (LCL equal/less than 60 days of lesion evolution), *Leishmania* patients present predominance in IL-4$^+$ and IL-10$^+$ T-lymphocytes. However, this is a transitory phenomenon, since patients with older lesions (LCL more than 60 days of lesion evolution) show a predominant Type-1 immune profile, suggesting that disease development may depend on a transient deregulation of T-cell response, during the initial phase of infection. Interestingly MST displayed a basal mixed Type-0 cytokines profile. However, the low frequency of IL-4$^+$ T-cells, high IFN-gamma$^+$/IL-10$^+$ cell ratio as well as elevated nitrite and nitrate plasma levels observed in MST, suggested that despite basal levels of cytokines, a high proportion of Type-1 over Type-2 cytokines would count to prevent parasite growth and lesion development.

2. INTRODUCTION

The clinical outcome of *Leishmania* infection in humans, ranging from relatively mild to severely life-threatening diseases depends on several host and parasite related factors. One of these factors is the strain of the infecting *Leishmania*. It has been demonstrated that a single strain of *Leishmania* can give rise to more than one clinical form of the disease (1). Differences in its clinical forms are likely to be influenced by the individual's immune response. The most prevalent clinical forms include cutaneous, mucocutaneous and visceral leishmaniasis (1). The cutaneous clinical (CL) form, caused by *Leishmania braziliensis* is characterized by the presence of a single or few ulcerated lesions with elevated borders, frequently located on the inferior limbs (2,3). These patients usually have partial immune resistance against the infection, leading to localized lesions (LCL), scarceness of parasites and a tendency of spontaneous healing or good response to antimonial therapy (4).

In murine models of leishmaniasis, there is evidence that the mechanisms of cure or resistance to *Leishmania major* infection are associated with macrophage activation, leading to the destruction of the parasite. In this context, Type-1 cytokines such as IFN-gamma and TNF-alpha have been demonstrated to play an important role in the process of macrophage activation and parasite destruction (5-8). On the other hand, the mechanisms of morbidity are related to the effects of Type-2 cytokines such IL-4, IL-10 and TGF-beta. These cytokines are able to inhibit the differentiation of Type-1 lymphocytes and the production of IFN-gamma and TNF-alpha. (5,8,9).

The human immune response to Leishmania infection is not as well characterized as in murine experimental models. Previous studies evaluating the immune response in human leishmaniasis, has been concentrated into two major aspects: the immunological mechanisms associated with disease progression and those associated with cure after etiological treatment. However, little is known about the immune response associated with natural resistance. Considering the immune mechanisms associated with CL progression, previous reports suggested an important role for IL-10 in the down-modulation of T-cell response during the early phase of infection, facilitating parasite multiplication and disease onset (10,11). Furthermore IL-4, which has been associated with macrophage deactivation and inhibition of the differentiation of Type-1 lymphocytes, has been associated with disease progression (12,13). In this context, Coutinho et al. (1998), showed a highly significant decrease in IL-4 production in the supernatants of antigen-stimulated PBMC cultures in cured patients when compared with the cellular response of the same patients during active disease.

In human cutaneous leishmaniasis, resistance acquired after spontaneous or chemotherapy-mediated clinical cure has shown an association with increased levels of IFN-gamma production (12-14). Indeed, subjects with self-healing CL have a stronger T-cell response and produce more IFN-gamma when compared with patients with active disease (15). Still focusing on protective immunomechanism, it has been observed that after vaccination, patients showing Montenegro skin test conversion, display increased frequency of CD8$^+$ T-cells and IFN-gamma production within *Leishmania*-reactive cells (16,17). Type-1 immune response, observed in these individuals suggested its role in protective immunity during human leishmaniasis, with IFN-gamma being the major cytokine involved in macrophage activation in association with TNF-alpha stimulating the synthesis of nitric oxide (NO) (18-20). Cytokine inducible NO synthase is one of the key defense mechanisms on mammalian phagocytes that have been demonstrated to be involved in the control of infection such as that caused by *Leishmania* (21). In leishmaniasis the preferential production of proinflammatory cytokines result in an increased synthesis of nitric oxide (NO) and reactive oxygen species, which are involved in host protection through direct on parasite toxicity or inhibition of parasite growth (22).

Although several studies have provided evidence for drug/vaccine induced resistance to infection, little is known about the events and the putative protective immune response associated with natural resistance in leishmaniasis. Subclinical *Leishmania* infection has been reported in areas where *Leishmania chagasi*, the causative agent of visceral leishmaniasis is endemic (23). Although no previous studies have been designed to evaluate the prevalence of subclinical *Leishmania braziliensis* infection, it has been reported that approximately 10% of healthy subjects have a positive skin test result for *Leishmania* antigen in areas where *L. braziliensis* is endemic (24,25). This finding suggests that there are subjects that may be able to control the disease. Therefore, study of the immune response of this group of individuals becomes critical for the understanding of protective immune response in human leishmaniasis.

Herein, we examined the intracellular cytokine profile of T-cells stimulated *in vitro* with soluble *Leishmania braziliensis* antigens (SLA), as well as the levels of plasma nitrite and nitrate in *Leishmania*-endemically exposed population. The immune response of patients with active LCL as well as non-infected individuals, both with and without positive Montenegro skin test and no history of previous LCL, were investigated to determine whether there were differences on the immune response in susceptible and resistant individuals. We believe that this approach may give us important information on the characteristics of susceptibility and a putatively protective immune response against cutaneous leishmaniasis in man.

3. MATERIAL AND METHODS

3.1. Study population

A written informed consent was obtained from all subjects included in this study, or from their parents or guardians. Treatment was offered to all infected individuals independently on their participation in the study. This study was approved by the Human Ethics Committee from the Fundação Oswaldo Cruz, Ministério da Saúde, Rio de Janeiro, Brazil.

Table 1. Characteristics of the study groups

Group	Abbreviation	Age	Number of Individuals		
			Men	Women	Total
Localized Cutaneous Leishmaniasis	LCL	5-58 (28.2)	17	11	28
Not infected with naturally positive Montenegro Test and no history of lesion or treatment	MST	12-71 (39.8)	10	5	15
Not Infected	NI	20-69 (34.7)	9	7	16

The ages are expressed (oldest and youngest) and mean in parenthesis

We evaluated three groups of individuals. The first consisted of 28 patients with active localized cutaneous leishmaniasis (LCL). The second consisted of 15 healthy individuals with positive Montenegro skin test, without history of lesion (MST), and the third consisted of 16 healthy individuals with negative Montenegro skin test that live in the same endemic area (NI). Characterization of the population is shown in Table 1. No differences were observed when the mean age, between the groups of individuals were analyzed (LCL: 28.2; NI: 34.7 and MST: 39.8; p= 0.075).

Stool exams were performed in all patients to identify helminth infections. Positive individuals were excluded from the study population. Diagnosis of leishmaniasis was based on dermatological findings, positive parasitological exams and a positive Montenegro skin test. Blood was collected immediately before beginning of treatment.

3.2. Montenegro's Skin Test

Montenegro's skin test (MST) was performed as previously described (26), using a standardized antigen preparation. Briefly, 4 μg (0.1 ml) of antigen was injected intradermally into the forearm. Readings were performed 48 hrs after injection. The presence of an enduration with a diameter of equal/more than 5mm was considered a positive result. MST was performed in individuals of all groups before blood collection.

3.3. Antigen preparation

Stationary phase (7 days) of *Leishmania V. braziliensis* (MHOM/BR/2903) was harvested from LIT medium, and washed 3 times in sterile PBS by centrifugation at 1,000 x g for 10 minutes at 4ºC. The cells were than disrupted by 3 cycles of sonication – 1 minute, 20 Hz (Ultrasonic Disrupter – Tekmar Cº - Cincinnati – OH). The suspension was sedimented at 4,000 x g for 10 min. The supernatant was collected, dialyzed and used as antigen for the studies of the immune response. Protein was quantified using method previously described (27). Soluble *Leishmania* antigen (SLA) was used at final concentration of 25 μg/mL in cell culture *in vitro*.

3.4. Monoclonal antibodies

In this study, we evaluated different cell phenotypes using anti-human Fluorescein Isothiocyanate (FITC) labeled monoclonal antibodies (mAbs) at a concentration of 0.5μg/ml, including isotypic control (clone X40), anti-CD3 (clone Leu-4), anti-CD4 (clone SK3) and anti-CD8 (clone SK1) all purchased from Becton Dickinson (San Jose, CA). Anti-human cytokines Phycoerythrin-labeled (PE) mAbs, anti-IFN-gamma (clone B27), anti-TNF-alpha (clone MAB11), anti-IL-4 (clone MP4-25D2) and anti-IL-10 (clone JES3-9D7) were

purchased from Pharmingen (San Diego, CA) and used at a final concentration of 25μg/ml.

3.5. *In vitro* short-term whole blood culture

Peripheral blood samples from 59 individuals were collected into Vacutainer tubes containing sodium heparin (Becton Dickinson Vacutainer). In this study, analysis of cytokine profile was evaluated after a short-term incubation *in vitro*, in the presence of antigen specific stimulation with soluble *Leishmania* antigen (SLA). Antigen stimulated cell cultures were incubated for 6 hours at 37ºC in a 5% CO_2 humidified incubator in the presence SLA at a final concentration of 25μg/ml, followed by the addition of 10μg/ml Brefeldin A-BFA (Sigma Chemical Company – St Louis, MO), at a final concentration of 10μg/ml, followed by 4 hours of incubation to inhibit cytokine secretion, allowing them to accumulate in the Golgi apparatus, leading to higher percentages of cytokine-positive cells, which increases sensitivity for further analysis. Following incubation, cultures were treated with 2mM EDTA (Sigma) and kept at room temperature for 15 minutes, to stop the cellular activation. Stimulated cultures were further used for the detection of intracellular cytokine profile as described below.

Negative and positive control cultures were also performed in order to evaluate the sample viability. For this purpose, whole blood samples were respectively treated with 500μl of RPMI 1640 and RPMI 1640 plus Phorbol 12-Myristate 13-Acetate-PMA (Sigma) at a final concentration of 25ng/ml plus ionomycin (Sigma) of 1μg/ml plus BFA at 10μg/ml. Blood samples were incubated for 4 hours at 37ºC in a 5% CO_2 humidified incubator. Cytokine patterns observed in control cultures confirmed viability of all samples, as demonstrated by high levels of IFN-gamma[+] and TNF-alpha^{+} cells, in PMA-stimulated as compared to negative control cultures (data not shown).

3.6. Flow cytometric immunostaining for intracellular cytokine analysis

After EDTA treatment, whole blood culture samples were washed with 6ml of FACS buffer containing 0.015M Phosphate Buffered Saline-PBS, 0.5% Bovine Serum Albumin-BSA and 0.1% sodium azide (Sigma), by centrifugation at 600 x g for 7 minutes at room temperature and 1ml of FACS buffer added to the tubes. Two hundred μl aliquots were individually stained with the manufactures recommended amounts of mAbs anti-CD4-FITC and anti-CD8 –FITC for 30 minutes at room temperature in the dark. Stained samples were treated by gentle vortexing with 2ml of FACS Lysing Solution (Beckton Dickinson, San Jose, CA) for erythrocyte lysis. FACS Lysing Solution, containing formaldehyde was used to fix the leukocytes
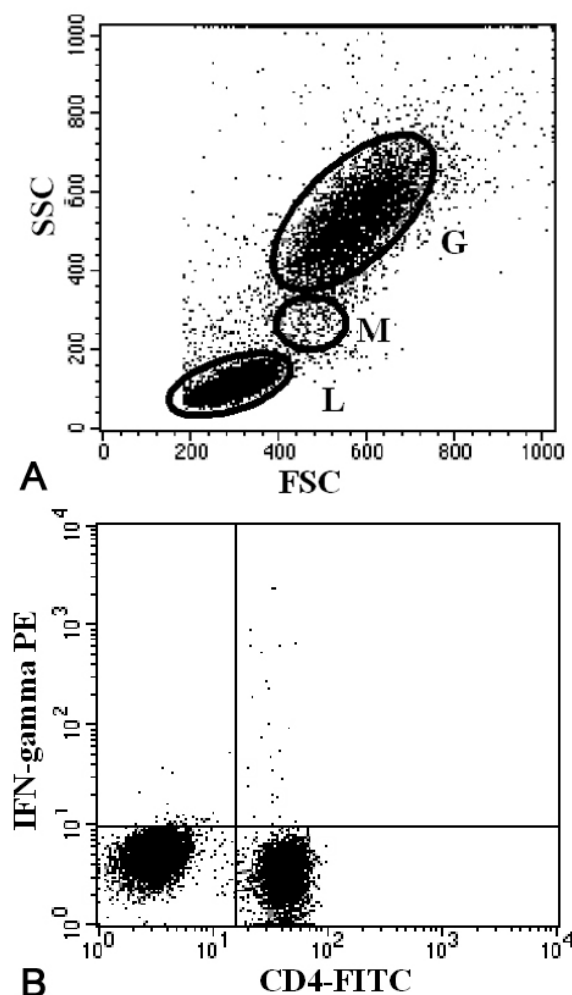
Figure 1. Identification of peripheral leukocytes populations in diagram of FSC x SSC (A). Dot plot of FL-1 x FL-2, displaying the frequency of CD4^{+} IFN-gamma^{+} cells, after the stimulation with SLA (B).

prior to staining for intracellular cytokines. The samples were then centrifuged at 600 x g for 7 minutes at room temperature, the supernatant was discarded and the cell pellet was ressuspended and kept in the dark for 10 minutes at room temperature with 2ml of FACS permeabilizing solution containing FACS buffer and 0.5% of saponin (Sigma). Following incubation, the samples were centrifuged at 600 x g for 7 minutes at room temperature. After centrifugation, the cells were ressuspended in 200µl of FACS buffer solution and distributed in 30µl aliquots over 96 wells U bottom microtiter plates (Thomas 9383-A90) and stained by incubation for 30 minutes at room temperature in the dark with 20µl of PE-labeled anti-cytokine mAb (anti-IFN-gamma, anti-TNF-alpha, anti-IL-4 and anti-IL-10) previously diluted 1:50 in sterile FACS permeabilizing solution. The cell were washed fixed in 200µl of FACS fix solution (10 g/l paraformaldehyde, 10.2 g/l sodium cacodylate and 6.63 g/l sodium chloride, pH 7.2) and stored at 4ºC in the dark prior to the flow

cytometric analysis. A total of 30,000 events/tube were acquired using a FACScalibur® flow cytometer (Becton Dickinson) properly set up to measure forward (FSC), side (SSC) light scatters, FITC (FL-1) and PE (FL-2) fluorescence. CELLQuest[TM] software provided by the manufacture was used for data acquisition and analysis.

Identification of the peripheral leukocyte populations: granulocyte (G), lymphocyte (L) and monocyte (M) were obtained in SSC versus FSC diagrams (Figure 1A). After gating the lymphocyte population, the same was evaluated in dot plot graphic of FL1 (anti-CD4 or CD8-FITC) x FL-2 (cytokine-PE). (Figure 1B).

All results were first expressed as percentage of cytokine-positive T-cells, selected as described above. T-cells populations, was evaluated by the sum of CD4^{+} and CD8^{+} cells. Final data were expressed as absolute number of cytokine-expressing T-lymphocytes (cells/mm^{3} of peripheral blood). These values were calculated by taking the percentage of cells that express the cytokine of interest, upper right quadrant in Figure 1B multiplied by the absolute number leukocyte subpopulation obtained by hematological analysis (Table 2).

3.7. Evaluation of plasma nitrite levels
Nitrite was measured using the Griess reaction, a standard laboratory test used to quantify nitrite levels as an indirect measurement of nitric oxide (NO) production. The test is based on the generation of a nitrosanting species upon nitrite acidification. Briefly, nitrate reductase was added to 50µl of serum diluted 1:4 in distilled water. After an overnight incubation at 37ºC, the samples were deproteinized by adding 1/20 volume of zinc sulfate (300g/L) to produce a final concentration of 15g/L. After, 100µl of Griess reagent (1g/L Sulfanilamide, 25g/L phosphoric acid and 0.1g/L N-1-naphthylenediamine) was added to the supernatant and incubated for 10 min, at room temperature and absorbance measured at 540nm. Each sample was assayed in duplicated. The concentration of Nox [sum of nitrites (NO_2^-) and nitrates (NO_3^-)] in the serum samples was determined by interpolation from a standard curve constructed by the dilution of sodium nitrate solution (linear range 0-100µmol/L) and plotting absorbance values for each standard against the corresponding concentration (28-30).

3.8. Statistical analysis
Statistical analysis was performed using non-parametric Kruskall-Wallis and Dunn's Multiple Comparison Test provided by the software GraphPad Prism, version 3.0 (San Diego, CA). Statistical significance was considered when p less than 0.05.

4. RESULTS

4.1. Unaltered Leukometry is observed in LCL and MST individuals
Absolute numbers and percentage of leukocyte subpopulations were determined from LCL patients, MST and NI group. No significant differences between the groups studied were observed in the mean values of

Table 2. Hematological status of the study population

Group	N	Leukocytes	Neutrophils	Eosinophils	Monocytes	Lymphocytes
NI	16	7,310 +/- 1,653	3,683 +/- 1,304 (48.0%)	486 +/- 272 (6.0%)	563 +/- 312 (8.7%)	2,458 +/- 811 (33.1%)
MST	15	6,589 +/- 1,512	3,441 +/- 931 (52.4%)	398 +/- 326 (5.7%)	457 +/- 233 (7.0%)	2,298 +/- 829 (34.9%)
LCL	28	6,966 +/- 2,300	4,080 +/- 1,587 (58.9%)	574 +/- 609 (7.5%)	511 +/- 272 (7.5%)	1,935 +/- 954 (28.0%)

The results are expressed as mean absolute number/mm^3 +/- SD and mean percent in parenthesis.
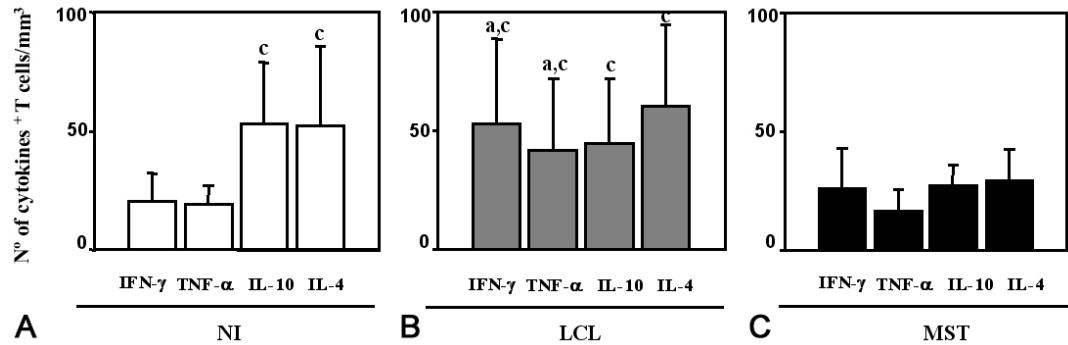

Figure 2. Cytokine expression by T-cells, evaluated by flow cytometry after a short *in vitro* stimulation with soluble leishmania antigen (SLA) as described in Material and Methods. a) Cytokine patterns in endemic non-infected individuals (NI). b) Patients with localized cutaneous leishmaniasis (LCL) and c) Individuals with naturally positive Montenegro skin test and no history of lesion (MST). The results are expressed as mean values of cytokine-positive gated T-lymphocyte/ mm^3 of blood +/- SD. The difference between the groups are considered significant at p less than 0.05 and are represent by letters: a = different when compared to NI group and b= different when compared to MST individuals.

circulating leukocyte, including white blood cells as well as neutrophils, eosinophils, monocytes and lymphocytes counts (Table 2).

4.2. Mixed Type-1 and Type-2 cytokines profile is observed in LCL and MST individuals

In order to understand the immune mechanisms associated with natural resistance, and those that take place during active infection, we evaluated, at a single cell level, the cytokine patterns of T-cells, after *in vitro* antigenic stimulation, as described in Materials and Methods. T-cell population was evaluated by the sum of CD4$^+$ and CD8$^+$ cells. Both subpopulations contributed for the cytokine profile observed. However, the CD4$^+$ subpopulation was observed to be the main source of the cytokines detected. Similar results were previously published by other authors (31).

Type-1 cytokines, such as IFN-gamma and TNF-alpha, are usually associated with activation of cellular response and are essential to induce macrophages leishmanicidal activity. Thus, it was of interest to investigate the expression of these cytokines in all subjects evaluated. Our results show a significant increase in the number of IFN-gamma$^+$ T-cells/mm^3 in LCL patients when compared to MST and NI groups (p less than 0.01 and p less than 0.05, respectively) (Figure 2). Similar increase was observed on the number of TNF-alpha$^+$ T-cells/mm^3 in LCL patients when compared to MST and NI groups (p less than 0.01 and p less than 0.05, respectively) (Figure 2). No differences were observed between MST and NI groups. When we compare the control and the SLA stimulated cultures, we observe an increase in the number of IFN-gamma and TNF-alpha$^+$ Tcells/mm^3 in specific stimulated cultures of LCL individuals (IFN-gamma - control: 25.5;

SLA: 53.0) (TNF-alpha - control: 15.1; LSA: 41.5). No differences were observed in NI groups (IFN-gamma - control: 16.8; SLA: 20.4) (TNF-alpha - control: 17.3; SLA: 19.5) and MST individuals IFN-gamma - control: 27.0; SLA: 25.9) (TNF-alpha - control: 20.5; SLA: 16.3).

An important cytokine involved in the control of the immune response to *Leishmania* infection is IL-10. It is well known that this cytokine plays and important role in the regulation of the immune response and inhibition of macrophage activity. Analysis of the expression of IL-10 by peripheral T-cells is shown in Figure 2. It is interesting to note that a significantly lower number of IL-10$^+$ T-cells were observed in the MST group when compared to both LCL and NI groups (p less than 0.01 and p less than 0.01, respectively). No differences were observed between LCL and NI groups. No significant differences were observed when we compared the control and specific stimulated cultures from the individuals evaluated (NI- control: 49.6; SLA: 53.2 / LCL – control: 36.1; SLA: 44.5 / MST- control: 24.3; SLA: 26.8).

IL-4 is an important cytokine that has been shown to deactivate inflammatory macrophages and to regulate the induction of Type-2 cells in addition to inhibit the development of Type-1 cells. Figure 2 shows that the number of T-cells expressing IL-4 is significantly lower in the MST group when compared to LCL and the NI groups of individuals (p less than 0.01 and p less than 0.05, respectively). No differences were observed between LCL and NI groups**.** No significant differences were observed when we compared the control and specific stimulated cultures from the individuals evaluated (NI- control: 41.7; SLA: 52.2 / LCL – control: 51.0; SLA: 60.6 / MST - control: 26.4; SLA: 29.3).
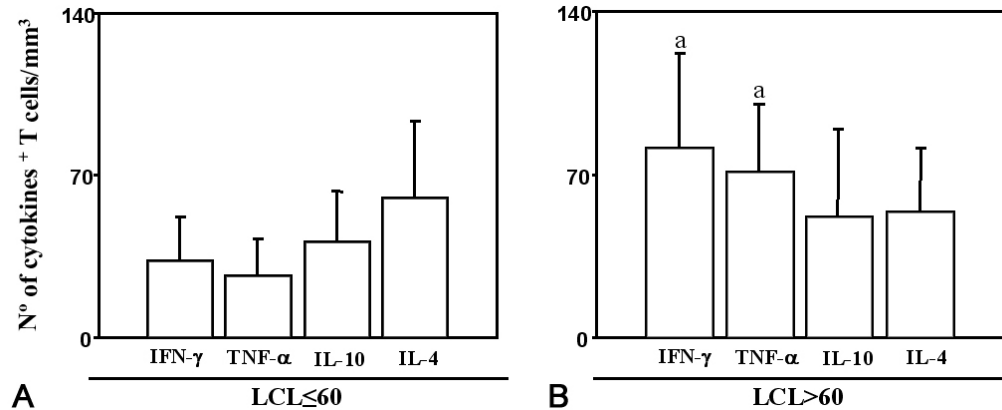
Figure 3. Cytokine expression by T-cells, evaluated by flow cytometry after a short soluble leishmania antigen (SLA) stimulation as described in Material and Methods. a) Cytokine patterns of localized cutaneous leishmaniasis patients with early lesions (LCL equal/less than 60 days of lesion evolution). b) Localized cutaneous leishmaniasis patients with old lesions (LCL more than 60 days of lesion evolution). The results were expressed as mean values of cytokine-positive gated T-lymphocyte/ mm^3 of blood +/- SD. The difference between the groups were considered significant at p less than 0.05 and are represent by letter a.

Taken together, our findings demonstrated a distinct mixed Type-0 cytokine patterns in LCL and MST individuals. When we evaluated the T-cell cytokine profile of LCL patients, we observed a mixed Type-0 immune response characterized by enhanced expression of both Type-1 cytokines (IFN-gamma and TNF-alpha) as well as Type-2 cytokines (IL-4 and IL-10) (Figure 2B). On the other hand, the analysis of the T-cell cytokine profile of MST individuals, despite of showing a mixed Type-0 immune profile, demonstrated a distinct pattern in comparison to the LCL group, with the latter displaying a significantly higher number of cytokine positive cells (Figure 2C).

Interestingly the NI group showed a completely different profile with a predominating Type-2 immune response, with higher percentages of IL-4+ and IL-10+ cells, after SLA stimulation (Figure 2A).

It is remarkable to notice that a mixed type of immune response (Type-1 and Type-2) may reflect the distinct phenomena at individual level including a mixed cytokine profile or the existence of subgroups with some individuals presenting a Type-1 and another displaying a Type-2 immune profile. This issue was further evaluated by sub grouping the LCL patients based on the time of lesion development as well as by calculating the cytokine ratios for the MST group.

4.3. A Type-2 immune response is observed in the early stage of human cutaneous leishmaniasis
The data presented above showed that patients with active infection develop a Type-0 immune response. However, previous work showed differences in the cytokine profile when patients with early lesions were compared with those with older lesions (10,11). To study further the cellular immune response triggered by *Leishmania* infection, and to elucidate the dynamics of the immunological events during active infection, we evaluated the T-cell cytokine pattern of LCL patients with different time of lesion development, following the antigen-specific stimulation *in vitro*. For these analyses the LCL group was subdivided in LCL patients with early lesions, with less than 60 days of evolution and patients with older lesions, with more than 60 days of evolution, based on previous described work (10), so we have also divided the study population using the same criteria.

Our results show that a distinct pattern of response was observed in LCL patients with early lesions when compared to the LCL patients with older lesions. LCL equal/less than 60 days displayed a typical susceptibility phenotype with the predominance of IL-4 and IL-10+ T-cells (Figure 3A). On the other hand, the group LCL more than 60 days showed a predominant Type-1 immune profile, with increased number of IFN-gamma and TNF-alpha+ T-cells (Figure 3B).

4.4. A Type-1 immune response is observed in the late stage of human cutaneous leishmaniasis
As disease progress, there was a clear switch from a Type-2 to a predominantly Type-1 immune response, which is consistent with the development of a cell mediated immunity that may be involved in clearing the infection. Our results show a significant increase in the number of IFN-gamma+ T-cells/mm^3 in LCL patients with older lesions when compared to patients with early lesions (p less than 0.05) (Figure 3). Similar increase in the number of TNF-alpha+ T-cells/mm^3 in LCL patients with old lesions was observed when compared to LCL patients with early lesions (p less than 0.01) (Figure 3). No differences were observed between the groups when we evaluated the expression of IL-10 and IL-4.

4.5. Type-1/Type-2 cytokines ratio in MST individuals pointed out for a predominant Type-1 immune profile.
Recently, it has being demonstrated that in humans the determining factor in the progression and
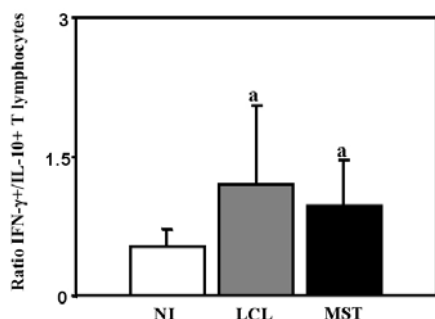
Figure 4. Mean percentage of IFN-gamma[+] T-cells/ IL-10^{+} T-cells +/- SD in patients with localized cutaneous leishmaniasis (LCL), individuals with naturally positive Montenegro skin test and no history of lesion (MST) and non-infected individuals (NI). The difference was significant at p less than 0.05 and are represent by letters: a = different when compared to NI.

Figure 5. Mean nitrite plus nitrate concentrations +/- SD in plasma of patients with localized cutaneous leishmaniasis (LCL), individuals with naturally positive Montenegro skin test and no history of lesion (MST) and non infected individuals (NI). The difference are significant at p less than 0.05 and are represent by letters: a = different when compared to NI group.
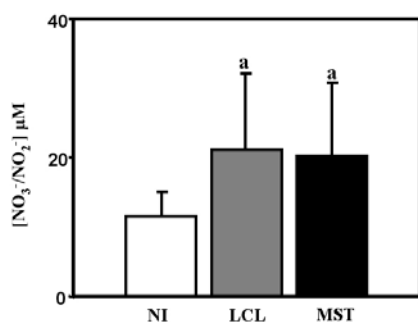
control in infectious disease is not the polarization of the immune response, but rather the balance between Type-1 and Type-2 immune response. Herein, we evaluated the balance between a Type-1 and Type-2 immune responses in all groups of individuals by analysis of cytokine ratios, focusing mainly on IFN-gamma and IL-10. Our results shows (Figure 4) an increased on the IFN-gamma/ IL-10 ratio in LCL and MST individuals when compared to NI group (p less than 0.05). No differences were observed between LCL and MST groups. It is interesting to note that the MST group despite of showing a mixed Type-0 cytokine profile by T-lymphocytes, presented a predominant Type-1 immune response when IFN-gamma/ IL-10 ratio was evaluated.

4.6. Individuals with active LCL and those of the MST group have high concentration of plasma nitrite plus nitrate levels.

In leishmaniasis, the preferential production of Type-1 cytokines results in an increased synthesis of Nitric oxide (NO) and reactive oxygen species by macrophages.

This mechanism has been demonstrated to be involved in host protection by direct toxicity on parasites or by inhibiting parasite growth (21). NO has a very short half-life, and within seconds after being produced, it is converted to nitrite and nitrate, sequentially, in the presence of molecular oxygen. Therefore, determination of plasma nitrite plus nitrate concentration reflects NO produced by the activity of iNOS (30). The nitrite plus nitrite concentration in the plasma were determined using the Griess method. As shown in Figure 5, plasma nitrite plus nitrate concentration were significantly higher in the LCL and MST groups when compared to NI individuals (p less than 0.01 and p less than 0.01, respectively). These results are expected in the LCL group, since they present a greater expression of Type-1 cytokines, and these are associated with macrophage activation, NO production and intracellular parasite killing. The results observed in MST group are very interesting. In this group we observed an increase in plasmatic nitrite plus nitrate concentration, but we did not observe an increased expression in Type-1 cytokines in T-lymphocytes. Since, there is a predominance of a Type-1 immune response, as determined by IFN-gamma /IL-10 ratio, we believe that these subjects who did not develop the disease, may have the ability to modulate their immune response, and the basal levels of IFN-gamma and TNF-alpha observed in these individuals are enough to activate macrophages and stimulate NO synthesis.

No significant difference were observed between LCL patients with early lesions and LCL patients with older lesions when we evaluated the plasmatic nitrite plus nitrate concentrations (16.7μM and 24.5μM, respectively).

5. DISCUSSION

Studies related to the analysis of the role of the host immune response on the pathogenesis and development of resistance to *Leishmania* has largely been made in inbred mice that are either susceptible or resistant to disease caused by *L. major*. However, the relationship between findings in the experimental model and human *Leishmania* infection has yet to be clearly established.

Investigations on human cutaneous leishmaniasis have involved patients with active disease, comparing different disease presentations, with healthy uninfected controls or patients that have had the lesions healed (12-14,31). In these studies susceptibility to infection was evaluated in individuals that are infected, resistant and those that have recovered from an infection mediated or not by treatment (4,15). However, little is known about the immune mechanisms associated with natural resistance to infection. In order to understand the immune mechanisms associated with natural resistance and those that take place during active infection, we have analyzed, at a single cell level, the cytokine patterns of T-cells, after a short in vitro antigen specific stimulation, as well as the plasma levels of nitrite and nitrate in susceptible infected individuals with localized cutaneous leishmaniasis (LCL), subjects with positive Montenegro skin test without history of lesion and treatment (MST), and endemic non-infected individuals with negative Montenegro skin test (NI).

In this study we observed that infected patients with localized cutaneous leishmaniasis present both Type-1 and Type-2 (Type-0) response by T-cells with the expression of IFN-gamma, TNF-alpha and IL-4 as well as of IL-10 cytokines (Figure 2B). LCL patients presented a significant increased in the number of IFN-gamma[+] and TNF-alpha[+] T-cells in comparison to non-infected individuals (MST and NI group) (Figure 2B). These observations are in agreement with previous works (4,31-32) where these cytokines have been associated with cure and resistance with IFN-gamma being the major cytokine involved in macrophage activation that together with TNF-alpha stimulates the synthesis of nitric oxide, considered to be major product involved in *Leishmania* killing (33) and resistance to *Leishmania* infection (15,34).

As mentioned above, nitric oxide has been associated with resistance to infection. Previous work showed that in patients with cutaneous leishmaniasis the plasma nitrite plus nitrate concentrations were higher than those of healthy subjects (35). A study in literature using immunohistochemistry showed that in the skin lesions of patients with cutaneous leishmaniasis expression of inducible nitric oxide synthase is also detectable (36). Our study showed an increased in the synthesis of IFN-gamma and TNF-alpha by lymphocytes (Figure 2B), together with an increase in plasmatic nitrite and nitrate (Figure 5) in the LCL group of individuals, suggesting that these patients can develop an immune response capable of killing intracellular *Leishmania*. In addition to mounting an effective Type-1 immune response against leishmaniasis, other studies suggest that the immunoregulation of the subsequent inflammatory response is important and necessary for maintaining host tissue integrity. An exacerbated Type-1 response has been associated with the development of mucosal leishmaniasis (37). However, our data show that in LCL patients both Type-1 and Type-2 cytokines are detectable in infected patients (Figure 2B). It is well known that synthesis of Type-2, as well as regulatory cytokines are important for the modulation of the Type-1 exacerbated immune response and that this modulatory activity is pivotal for the maintenance of tissue integrity in the host. In this context a positive correlation between SLA specific IFN-gamma production and the number of IL-10 producing lymphocytes in LCL patients was demonstrated (38,39). Our data and those of others (15,32) show that the expression of IFN-gamma and TNF-alpha may be associated with *Leishmania* killing in LCL patients. This is also suggested by the observation of significantly higher level of plasmatic nitrite and nitrate (Figure 5). However, it is not clear which is the mechanism leads to parasite survival in these patients, since the cytokine environment favors the killing of the intracellular *Leishmania*. One possible explanation is that the development of disease may depend on a transient deregulation of T-cell response during the initial phase of infection. In experimental models of leishmaniasis it has been demonstrated that cytokines secreted in the early phase of the infection are important to determine its progression or control. In human infection some differences were observed on the cytokine profile when patients with newer lesions were compared to those with older lesions (10,11). Based on these studies, it was hypothesizes that alterations on the immunological response early in human infection may allow parasite multiplication and clinical outcome of the disease. In fact, our data show that early in the infection (less than 60 days) patients with LCL present a typical susceptibility phenotype with a characteristic Type-2 response (Figure 3A), with the predominance of IL-4^{+} and IL-10^{+} T-lymphocytes. The predominance of these cytokines may allow parasite survival and multiplication, leading to the development of disease. However, our results suggest that the early Type-2 response is a transitory phenomenon. This is supported by the observation that patients with older lesions (more than 60 days) show a predominant Type-1 immune profile, with a significant increase on the expression of IFN-gamma[+] and TNF-alpha[+] T-lymphocytes when compared to individuals with early lesions (Figure 3B). Similar results were previously described (10,11). These authors, as in the current study, also evaluated the role of IFN-gamma and IL-10 on the immune response to *Leishmania* infection. It was suggested that the T-cell response during the early-phase of *Leishmania* infection is down-regulated by IL-10 and may facilitate parasite multiplication. Considering these results and those presented in this report we hypothesize that in susceptible individuals, early after exposure, a Type-2 immune response is triggered allowing parasite growth and survival and, as the disease progresses, this response changes to a Type-1 immune response, which activates macrophages leading to control of parasite growth and consequently disease progression.

It is well known that in endemic areas for *Leishmania*, there is a group of individuals that, although living in these areas, do not develop disease but have positive Montenegro skin test. This group of individuals had lower numbers of IFN-gamma, TNF-alpha, IL-10 and IL-4^{+} T-cells (Figure 2C). In these individuals both Type-1 and Type-2 cytokines were detected suggesting, once again, that the outcome of the clinical form or resistance to an infection is not directly linked to one cytokine profile but rather to the balance of these factors. These subjects who did not develop disease may have the ability to develop an effective immune response early after infection. However, these responses, mainly related to the levels of IFN-gamma and TNF-alpha are not necessarily high but are sufficient to control parasite growth and eliminate the *Leishmania* without inducing pathology. This hypothesis is further supported when we evaluate IFN-gamma/IL-10 ratios (Figure 4). It can be observed that in this group a significantly difference in this ratio as well as of nitrite and nitrate plasma levels is detected (Figure 5) when compared to NI group, and similar to that observed in the infected LCL group. Previous study evaluated the immune response of a group of individuals where the disease is considered to be subclinical. This group was composed of subjects who had converted Montenegro skin test and remained assymphomatic during the 4 years of follow-up. In this group of subclinical individuals, similar to our data, the levels of IFN-gamma and TNF-alpha were lower than in LCL patients. However mean IL-5 levels were slightly higher in individuals with subclinical infection than in LCL patients (40). The authors suggest that individuals who do

not develop disease when infected with *L. braziliensis* may have the ability to modulate their immune response. Our data confirm this hypothesis, since in our studies we have also observed a decrease in the synthesis of IL-4 and IL-10 by T-cells in MST when compared to LCL and NI individuals. We believe that the main cytokine associated with the modulation of these responses is IL-10, an important cytokine with modulatory activity exhibiting human macrophages deactivating properties. Therefore, in the presence of low numbers of IL-10$^+$ T-lymphocytes, the basal levels of IFN-gamma and TNF-alpha are sufficient to activate macrophages, stimulate NO synthesis and killing of intracellular *Leishmania*, without the development of skin lesion.

Together the data presented in this paper demonstrate that although there are significant differences on the immune response of the individuals evaluated, the determining factor related to the development of resistance or susceptibility to the infection is related to the balance of the levels of the different cytokine. This balance has been suggested, in other diseases, such as schistosomiasis (41) and Chagas disease (42), to be crucial in determining the outcome of the infection.

6. REFERENCES

1. Liew F.Y & C. A. O'Donnell: Immunology of leishmaniasis. *Adv Parasitol* 32, 161-259 (1993)

2. Llanos-Cuentas E.A, C. C Cuba, A. C. Barreto & P. D. Mardsen: Clinical characteristics of human *Leishmania braziliensis* infection. *Trans R Soc Trop Med Hyg* 78, 845-846 (1984)

3. Jones T. C, W. D. Johnson, A. C. Barreto, E. Lago, R. Badaro, B. Cerf, S. G. Reed, E. M. Netto, M. S. Tada & T. F. Franca: Epidemiology of American cutaneous leishmaniasis due to *Leishmania braziliensis braziliensis*. *J Infect Dis* 156, 73-83 (1987)

4. Da-Cruz A. M, R. Bittar, M. Mattos, M. P. Oliveira-Netto, R. Nogueira, V. Pinho-Ribeiro, R. B. Azeredo-Coutinho & S. G. Coutinho: T-cell-mediated immune response in patients with cutaneous or mucosal leishmaniasis: Long-term evaluation after therapy. *Clin Diag Lab Immunol* 9, 251-256 (2002)

5. Heinzel F. P, M. D. SadicK, S. S. Mutha & R. M. Locksley: Production of interferon-gamma, interleukin-2, interleukin-4 and interleukin-10 by CD4+ lymphocytes *in vivo* during healing and progressive murine leishmaniasis. *Proc Natl Acad Sci USA* 88, 7011-7015 (1991)

6. Scott P: Host and parasite factors regulating the development of CD4+ subsets in experimental cutaneous leishmaniasis. *Res Immunol* 142, 32-36 (1991)

7. Coffman R. L, R. Chaletalin, L. M. C. C. Leal & K. Varkila: *Leishmania major* infection in mice: a model system for the study of CD4+ T-cell subset differentiation. *Res Immunol* 142, 36-40 (1991)

8. Sacks D. L & N. Noben-Trauth: The immunology of susceptibility and resistance to Leishmania major in mice. *Nat Ver Immunol* 2, 845-858 (2002)

9. Barral A, M. Barral-Netto, E. C. Yong, C. E. Brownell, D. R. Twardzik & S. G. Reed: Transforming growth factor beta as a virulence mechanism for *Leishmania braziliensis*. *Proc Natl Acad Sci USA* 90, 3442-3446 (1993)

10. Ribeiro-de-Jesus A, R. P. Almeida, H. Lessa, O. Bacellar & E. M. Carvalho: Cytokine profile and pathology in human leishmaniasis. *Braz J Med Biol Res* 31, 143-148 (1998)

11. Rocha P. N, R. P. Almeida, O. Bacellar, A. R. Jesus, D. Correia-Filho, A. C. Filho, A. Barral, R. L. Coffman, E. M. Carvalho: Down-regulation of Th1 type response in early human American cutaneous leishmaniasis. *J Infec Dis* 180, 1731-1734 (1999)

12. Coutinho S. G, M. P. Oliveira, A. M. Da-cruz, P. De-Luca, S. C. F. Mendonça, A. L. Bertho, L. Soong & D. McMahon-Pratt: T-cell responsiveness of American cutaneous leismaniasis patients to purified *Leishmania pifanoi* amastigotes antigens and *Leishmania braziliensis* promastigotes antigens: Immunologic patterns associated with cure. *Exp Parasitol* 84, 144-155, (1996)

13. Coutinho S. G, A. M. Da-Cruz, A. L. Bertho, M. A. Santiago & P. De-Luca: Immunologic patterns associated with cure in human American cutaneous leishmaniasis. *Braz J Med Biol Res* 31, 139-142 (1998).

14. Da Cruz A. M, F. Conceição-Silva, A. L. Bertho & S. G. Coutinho: Leishmania - reactive CD4+ and CD8+ T cells associated with cure of human cutaneous leishmaniasis. *Infect Immun* 62, 2614-2618 (1994)

15. Carvalho E. M, D. Correia-Filho, O. Bacellar, R. P. Almeida, H. Lessa & H. Rocha: Characterization of the immune response in subjects with self-healing cutaneous leishmaniasis. *Am J Trop Med Hyg* 53, 273-277 (1995)

16. Mendonça S. C. F, P. De-Luca, W. Mayrink, T. G. Restom, F. Conceição-Silva, A. M. Da-Cruz, A. L. Bertho, C. A. Costa, O. Genaro, V. P. C. P. Toledo & S. C. Coutinho: Characterization of human T lymphocyte-mediated immune responses induced by a vaccine against american tegumentary leishmaniasis. *Am J Trop Med Hyg* 53, 195-201 (1995)

17. De-Luca P. M, W. Mayrink, C. R. Alve, S. G. Coutinho, M. P. Oliveira, A. L. Bertho, V. P. C. P. Toledo,

C. A. Costa, O. Genaro & S. C. F. Mendonça: Evaluation of the stability and immunogenicity of autoclaved and nonautoclaved preparations of a vaccine against american tegumentary leishmaniasis. *Vaccine* 17, 1179-1185 (1999)

18. Nathan C. F, H. W. Murray, M. E. Wiebe & Y. Rubin: Identification of interferon gamma as the lymphokine that activates human macrophages oxidative metabolism and antimicrobial activity. *J Exp Med* 158, 670-689 (1983)

19. Liew F. Y, C. Parkinson, S. Millor, A. Sevem & M. Carrier: Tumor necrosis factor (TNF-alpha) in leishmaniasis. 1. TNF-alpha mediates host protection against cutaneous leishmaniasis. *Immunology* 69, 570-573 (1990)

20. Liew F. Y: Regulation of nitric oxide synthesis in infectious and autoimmune disease. *Immunol Lett* 43, 95-98 (1994)

21. Nathan C & M. U. Shiloh: Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci USA* 97, 8841-8848 (2000)

22. Brunet L. R: Nitric oxide in parasitic infection. *Int Immunopharmacol* 1, 1467-1475 (2001)

23. Carvalho E. M, A. Barral, J. M. Costa, A. Bittencourt & P. Mardsen: Clinical and immunophatological aspects of disseminated cutaneous leishmaniasis. *Acta Trop* 56, 315-325 (1994)

24. Badaró R, T. Jones, E. M. Carvalho, D. Sampaio, S. G. Reed, A. Barral, R. Teixeira & W. D. Jr. Johnson: New perspectives on a subclinical form of visceral leishmaniasis. *J Infect Dis* 154, 1003-1011 (1986)

25. Davies C. R, E. A. Llanos-Cuentas, S. D. Pyke & C. Dye: Cutaneous leishmaniasis in the Peruvian Andes: an epidemiological study of infection and immunity. *Epidemiol Infect* 114, 297-318 (1995)

26. Costa C. A, V. P. C. P. Toledo, O. Genaro, P. Williams & W. Mayrink: Montenegro skin test – evaluation of the composition and stability of the antigen preparation. *Mem Inst Oswaldo Cruz* 91, 193-194 (1996)

27. Lowry O. H, N. J. Rosenbrough, A. L. Farr & R. J. Randall: Protein measurement with the folin phenol reagent. *J Biol Chem* 193, 265-275 (1951)

28. Green L. C, D. A. Wagner, J. Glogowski, P. C. Skipper, J. S. Wishnok & S. R. Tannenbaum: Analyses of nitrate, nitrite and (^{15}N) nitrite in biological fluids. *Anal Biochem* 126, 131-138 (1982)

29. Moshage H, B. Kok, J. R. Huizenga & P. L. Jansen: Nitrite and nitrate determinations in plasma: a critical evaluation. *Clin Chem* 41, 892-896 (1995)

30. Archer S: Measurement of nitric oxide in biological models. *FASEB J* 7, 349-360 (1993)

31. Bottrel R. L. A, W. O. Dutra, F. A. Martins, B. Gontijo, E. Carvalho, M. Barral-Netto, A. Barral, R. P. Almeida, W. Mayrink, R. Locksley & K. J. Gollob: Flow cytometric determination of cellular sources and frequencies of key cytokine-producing lymphocytes directed against recombinant LACK and soluble leishmania antigen in human cutaneous leishmaniasis. *Infect Immun* 69, 3232-3239 (2001)

32. Kemp K, T. G. Theander, L. Hviid, A. Garfar, A. Kharazmi & M. Kemp: Interferon-gamma and tumor necrosis factor-alpha-producing cells in humans who are immune to cutaneous leishmaniasis. *Scan J Immunol* 49, 655-659 (1999)

33. Green S. J, R. M. Crawford, J. T. Hockmeyer, M. S. Meltzer & C. A. Nacy: Leishmania major amastigotes initiate the L-arginine-dependent killing mechanism in IFN-gamma-stimulated macrophages by induction of tumor necrosis factor-alpha. *J Immunol* 145, 4290-4997 (1990)

34. Sadick M. D, R. M. Locksley, C. Tubbs & H. V. Raft: Murine cutaneous leishmaniasis: resistance correlates with the capacity to generate interferon-gamma in response to Leishmania antigens in vitro. *J Immunol* 136, 655-661 (1986)

35. Erel O, A. Kocyigit, V. Bulut & M. S. Gurel: Reactive nitrogen and oxygen intermediates in patients with cutaneous leishmaniasis. *Mem Inst Oswaldo Cruz* 94, 179-183 (1999)

36. Qadoumi M, I. Becker, N. Donhauser, M. Röllinghoff & C. Bogdan: Expression of inducible nitric oxide synthase in skin lesions of patients with American cutaneous leishmaniasis. *Infect Immun* 70, 4638-4642 (2002)

37. Bacellar O, H. Lessa, A. Schriefer, P. Machado, A. Ribeiro-de-Jesus, W. O. Dutra, K. J. Gollob & E. M. Carvalho: Up-regulation of Th1-type responses in mucosal leishmaniasis patients. *Infect Immun* 70, 6734-6740 (2002)

38. Pompeu M. M. L, C. Brodskyn, M. J. Teixeira, J. Clarêncio, J. Van Weyenberg, I. C. B. Coelho, S. A. Cardoso, A. Barral & M. Barral-Netto: Differences in gamma interferon production in vitro predict the pace of the in vivo response to *Leishmania amazonensis* in healthy volunteers. *Infect Immun* 69, 7453-7460 (2001)

39. Antonelli L. R. V, W. O. Dutra, R. P. Almeida, O. Bacellar & K. J. Gollob: Antigen specific correlations of cellular immune responses in human leishmaniasis suggest mechanisms for immunoregulation. *Clin Exp Immunol* 136, 341-348 (2004)

40. Follador I, C. Araújo, O. Bacellar, B. C. Araújo, P. L. Carvalho, P. R. Almeida & E. M. Carvalho: Epidemiologic and Immunologic findings for the subclinical form of

Leishmania braziliensis infection. *Clin Infec Dis* 34, e54-e58 (2002)

41. Correa-Oliveira R, I. R. Caldas & G. Gazzinelli: Natural versus drug-induced resistance in Schistosoma mansoni infection. *Parasitol Today* 16, 397-399 (2000)

42. Gomes J. A, L. M. Bahia-Oliveira, O. A. Martins-Filho, G. Gazzinelli & R. Correa-Oliveira: Evidence that development of severe cardiomyopathy in human Chaga's disease is due to a Th1-specific immune response. *Infect Immun* 71, 1185-1193 (2003)

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