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Flow cytometric analysis of cellular infiltrate from American tegumentary leishmaniasis lesions

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Summary

Background CD4+ and CD8+ T lymphocytes play different roles in the outcome of leishmaniasis. However, T-cell distribution in lesions shows significant variability in *in situ* immunocytochemical studies.

Objectives In this report flow cytometry was used to determine the predominant T-cell subsets in leishmaniasis lesions, and their relationship with *Leishmania*-responsive circulating T cells.

Patients and methods Mononuclear cells from lesions or peripheral blood (PBMC) of 34 cutaneous (CL), four mucosal (ML) and four disseminated leishmaniasis were phenotypically characterized by flow cytometry. *Leishmania*-responsive T cells were obtained after *in vitro* stimulation of PBMC with leishmanial antigens.

Results/conclusions Variable amounts of γδ lymphocytes were present in all lesions, with no association with duration of illness. The highest percentages of interleukin-2R- and interferon-γR-positive cells were observed in ML lesions and could render these T cells more susceptible to the effects of these cytokines. The distribution of intralesional T-lymphocyte subsets was quite variable (CD4+ > CD8+ = 18 cases, CD8+ > CD4+ = 12 cases and CD4+ ≅ CD8+ = 4 cases) without any association with clinical parameters, and could explain the controversy regarding proportions of these T-cell subsets in leishmaniasis lesions. Low percentages of *Leishmania*-reactive CD8+ T cells were observed in blood while an enrichment of CD8+ cells was shown in the inflammatory infiltrate, suggesting that local immunoregulatory factors could favour the recruitment and/or proliferation of local CD8+ lymphocytes. Increased percentages of CD8+ cells observed in older lesions are consistent with the hypothesis that they can mediate healing, although their involvement in tissue damage cannot be ruled out. It is possible that these mechanisms can influence the clinical outcome or even the response to therapy.

American tegumentary leishmaniasis (ATL) is a chronic inflammatory disease, which in Brazil is caused mainly by *Leishmania* (*Viannia*) *braziliensis*. After being introduced into the host by the bite of the sand fly the parasite interacts with the skin immune system, infecting local dendritic cells, macrophages and neutrophils. Because the hallmark of tissue inflammation is the recruitment, migration and activation of leucocytes, it is expected that the skin immune system plays a critical role in T-cell responses against many tegumentary diseases.[1–4]

The majority of patients develop cutaneous leishmaniasis (CL), a benign clinical form characterized by a single or a few localized skin ulcers, which usually respond promptly to antimonial therapy or even undergo spontaneous healing. A small number of patients present with disseminated leishmaniasis (DissL), where the ulcers are spread all over the skin surface.[5] Only 3–5% of *L. braziliensis*-infected individuals will develop mucosal leishmaniasis (ML), a severe disfiguring disease affecting mainly the oral or nasopharyngeal mucous membranes, which is usually more resistant to antimonial therapy.

The pathogenesis of leishmaniasis appears to be highly dependent on cellular-mediated immune responses, which have been proposed to influence the clinical outcome of disease either by T-lymphocyte subset effector functions or cytokine profile.[6–11] Notably, the magnitude of T-cell responses tends to be higher in ML than in CL patients, mainly in terms of the frequency of *Leishmania*-reactive T lymphocytes in lesions, parasite-specific cytotoxicity and also interferon (IFN)-γ and tumour necrosis factor-α production.[9–14] Previous studies have shown an association between active leishmaniasis and a predominance of *Leishmania*-reactive CD4+ over CD8+

Table 1 Clinical and laboratory main features of American tegumentary leishmaniasis patients studied

Clinical form	Age (years)	Sex (M : F)	Duration of illness (days)	Lesion size (cm^2)	MST (mm)	Positive NNN[a]	Serology for *Leishmania*
CL	34·9 ± 18·2	22 : 12	92 ± 58	10·7 ± 11·5	19·6 ± 12·6	28/29	15/34
ML	63·2 ± 3·2	2 : 2	684 ± 54	ND	22·7 ± 12·5	4/4	2/4
DissL	45·3 ± 5·1	4 : 0	130 ± 62	7·5 ± 2·7	11 ± 4·6	4/4	4/4

[a]a/b number of positive tests over the total number of cases analysed. CL, cutaneous leishmaniasis; ML, mucosal leishmaniasis; DissL, disseminated leishmaniasis; M, male; F, female; ND, not determined; MST, Montenegro skin test; NNN, McNeal Novy and Nicolle modified culture medium. Results are expressed as mean ± SD.

T cells derived from blood. After therapy, a decrease in CD4+ T cells occurs concurrently with an increase in CD8+ T cells, suggesting that CD8+ lymphocytes could be involved in the healing process.[7,8,10,15] These *Leishmania*-primed lymphocytes are recruited from blood into the inflammatory infiltrate. It is known that the frequency of specific T cells is much higher in leishmaniasis lesions than in blood.[13]

The dermal infiltrate of leishmaniasis consists mainly of lymphocytes, plasmocytes and histiocytes, and seems to be similar in both CL and ML.[16,17] Memory T lymphocytes predominate in leishmaniasis lesions.[18] Distinct histopathological pictures are not usually correlated with clinical and prognostic features.[19] In Brazil, different ratios of T-cell distribution in lesions have been reported: CD4+ > CD8+,[18,20] CD4+ = CD8+,[21] and CD4+ < CD8+.[22,23] Higher proportions of CD8+ T cells have been observed in CL caused by Old World[24] and New World *Leishmania* species.[2,25] In addition, patients from Venezuela[26,27] and French Guyana[28] showed similar CD4/CD8 levels in lesions. These data point to the heterogeneity of T-cell subset distributions in leishmaniasis lesions.[29]

In situ immunopathological studies on leishmaniasis have improved our understanding of the local immune response, by characterizing not only cellular composition but also microanatomic distribution within the skin inflammatory infiltrate.[18,20,23,29] In addition, it is known that histopathological patterns can differ within the same lesion, depending on the site of the ulcer or even the duration of the disease.[19] Besides this, many points still need to be clarified, including the nature of the T-cell subset that effectively predominates in lesions, and its relationship with *Leishmania*-specific circulating T cells. In this report, we used flow cytometry to determine the phenotype of mononuclear inflammatory infiltrate cells obtained from lesion fragments of patients with different clinical forms of *L. braziliensis* infection. Our aim was to investigate a possible relationship between clinical outcome and phenotype of lymphocytes that are infiltrating lesions.

Patients and methods

Patients

Forty-two ATL patients suffering from active CL (n = 34), ML (n = 4) or DissL (n = 4) were studied. The main clinical and laboratory features of the patients studied are described in Table 1. All had acquired the disease in endemic areas of *L. braziliensis* infection. The following criteria were used for diagnosis: (i) positive Montenegro skin test (MST)-delayed-type hypersensitivity to leishmanial antigens; (ii) presence of *Leishmania*-specific serum antibodies; (iii) detection of parasites by microscopic examination or culture; and (iv) histopathological picture. This work was conducted according to federal ethical guidelines (Conselho Nacional de Saúde, Ministério da Saúde, Brazil).

Extraction of cells from tissues

Incisional skin or mucosal biopsy was performed for diagnostic purposes. Cells were obtained from lesions as described elsewere.[30] In brief, the skin specimen, stripped of subcutaneous fat, was placed in a tissue sieve fitted with a 64-μm mesh filter. The tissue sieve was placed on a Petri dish containing RPMI 1640 supplemented with 10 mmol L^{-1} HEPES, 1·5 μmol L^{-1} L-glutamine and 0·04 mmol L^{-1} 2-mercaptoethanol (Sigma Chemical Co., St Louis, MO, U.S.A.). The tissue was cut into pieces and extruded through the mesh using surgical scalpels. The single-cell suspension was washed once and the mononuclear cells separated by centrifugation over a Ficoll–Hypaque gradient (Histopaque 1077; Sigma). In parallel, peripheral blood mononuclear cells (PBMC) were also obtained as described elsewere.[10] Mononuclear cells obtained from lesion (LMC) and blood were re-suspended in cold phosphate-buffered saline (PBS) containing 0·01% sodium azide (PBSaz).

Flow cytometric immunophenotyping

LMCs or PBMCs were adjusted to 10^6 cells per 200 μL in PBSaz plus formalin 0·1% and incubated for 30 min at 4 °C in the presence of 5 μL of each monoclonal antibody (mAb). The following mAbs were used: anti-CD3, anti-CD19, anti-CD4, anti-CD8, anti-CD14, anti-CD56, anti-CD25 (Beckman-Coulter, Hialeah, FL, U.S.A.), anti-IFN-γR (Genzyme Corp., Cambridge, MA, U.S.A.) and TCRγ1 (Identi-T; T Cell Diagnosis, Inc., Cambridge, MA, U.S.A.). After 30 min of staining incubation, the cells were washed three times prior to analysis by flow cytometry.

Ten thousand events were acquired in each sample run and the data were analysed with EXPO32™ software in an EPICS ALTRA flow cytometer (Beckman-Coulter).

Phenotypic characterization of *Leishmania braziliensis*-reactive T cells

Leishmania antigen-reactive T cells were obtained after *in vitro* stimulation of PBMC (3×10^6 per well) in the presence of the equivalent of 5×10^6 disrupted *L. braziliensis* (Lb) promastigotes under the conditions described above. After 5 days in culture, the Lb-reactive T cells were harvested and washed, then separated by centrifugation over discontinuous Percoll gradient (Sigma). The Lb-reactive blast T cells were stained with anti-CD3, anti-CD4 and anti-CD8 mAbs and analysed by flow cytometry as described above.

Statistical analysis

Mann–Whitney U-test or Spearman rank correlation were applied using GraphPad Instat™ V2.04 software (GraphPad™ Software, San Diego, CA, U.S.A.).

Results

T lymphocytes were the predominant mononuclear cell population in all clinical forms studied when compared with macrophages, B or γδ lymphocytes (Table 2). The percentage of T cells (mean ± standard error) was higher in ML (80·2 ± 5·7%) than in CL (55·8 ± 4·3%) or DissL (51·1 ± 14·4%) patients. The proportion of CD4+ and CD8+ T cells was similar among CL patients (CD4/CD8 ratio = 1·16 ± 0·11). In ML and DissL the CD4/CD8 ratio was 1·52 ± 0·4 and 0·66 ± 0·1, respectively, although those differences were not statistically significant. There was a great variability in the distribution of CD4+ and CD8+ T lymphocytes within the lesions (Fig. 1). In CL patients, CD4+ T lymphocytes infiltrating the lesions predominated over CD8+ T cells in 18 cases, while CD8+ T cells were the dominant T-cell subpopulation in 12 cases. Only four cases presented similar proportions of CD4+ and CD8+ T cells.

B cells were present in all lesions studied. γδ lymphocytes were present in a higher proportion in ML than in CL or DissL lesions. Macrophages were present in variable percentages in all clinical forms.

The percentage of cells expressing interleukin (IL)-2 receptor ($P = 0{\cdot}02$) as well as IFN-γ receptor was much higher in ML than in CL patients. Only DissL patients presented lower levels of IL-2R in comparison with ML or CL patients (Table 2).

There was no correlation between the proportion of mononuclear cells and clinical parameters such as the size of lesion or even MST intensity.

In CL patients the percentages of CD4+ ($P = 0{\cdot}01$, $r = 0{\cdot}41$) and CD8+ ($P = 0{\cdot}02$, $r = 0{\cdot}37$) lymphocytes were positively correlated with the duration of illness (Fig. 1). The mean period of illness was 92 days; patients with older lesions (more than 3 months, 10 cases) showed high proportions of CD4+ (35·2% ± 2·6%) and CD8+ (32·1% ± 3%) T cells. Recent lesions (less than 3 months' duration) presented lower proportions of CD4+ (26·6% ± 2·1%) and CD8+ (27·7% ± 2·3%) T cells. As ML and DissL are not frequent clinical forms, the low number of cases analysed did not allow an association with disease duration.

The percentages of CD4+ or CD8+ T cells were compared in PBMC and lesions of 17 patients. The CD4/CD8 ratios in PBMC were similar to those observed in the healthy population.[31] The mean (± SE) proportion of CD4+ T cells was lower in the lesions (30·2% ± 2·9%) than in blood (38·4% ± 2%) ($P = 0{\cdot}002$). Conversely, CD8+ T-lymphocyte percentages were significantly higher ($P = 0{\cdot}03$) in lesions (31·1 ± 1·3%) in comparison with blood (23·1 ± 3%). As PBMCs include T lymphocytes that recognize different types of antigens, we decided to investigate if there was a relationship between the percentages of T-cell subsets infiltrating leishmaniasis lesions and the proportions of CD4+ and CD8+ blast T cells obtained after *in vitro* stimulation of PBMCs with *Leishmania* antigens. Twelve patients were included in this study. In accordance with our previous results,[7] nine of 12 patients presented higher percentages of CD4+ than CD8+ *Leishmania*-reactive T cells in PBMC. There was no significant association between the proportions of these *Leishmania*-reactive T-cell

Table 2 Phenotypic characterization assessed by flow cytometry of mononuclear cells derived from lesions of American tegumentary leishmaniasis patients

Phenotypic characterization	Cutaneous leishmaniasis	n	Mucosal leishmaniasis	n	Disseminated leishmaniasis	n
T	55·8 ± 4·3	20	80·2 ± 5·7	03	51·1 ± 14·4	02
B	10·6 ± 1·9	10	22·1	01	9·5 ± 3·3	02
T CD4	29·9 ± 1·7	34	35·1 ± 4·6	04	24·1 ± 3·6	04
T CD8	28·9 ± 1·9	34	27·5 ± 5·8	04	39·4 ± 10·3	04
γδ	7·5 ± 1·2	16	10·5 ± 1·9	03	4·8 ± 0·8	03
Macrophages	15·7 ± 3·9	14	29·5 ± 13·5	03	9·9 ± 2	03
IL-2 receptor	14·0 ± 0·6	04	87·2 ± 5·2	03	9·6	01
IFN-γ receptor	56·1 ± 9·2	04	81·3 ± 6·6	03	ND	ND

Results expressed as mean (%) ± standard error. IL, Interleukin; IFN, interferon; ND, not determined. Number of patients analysed.

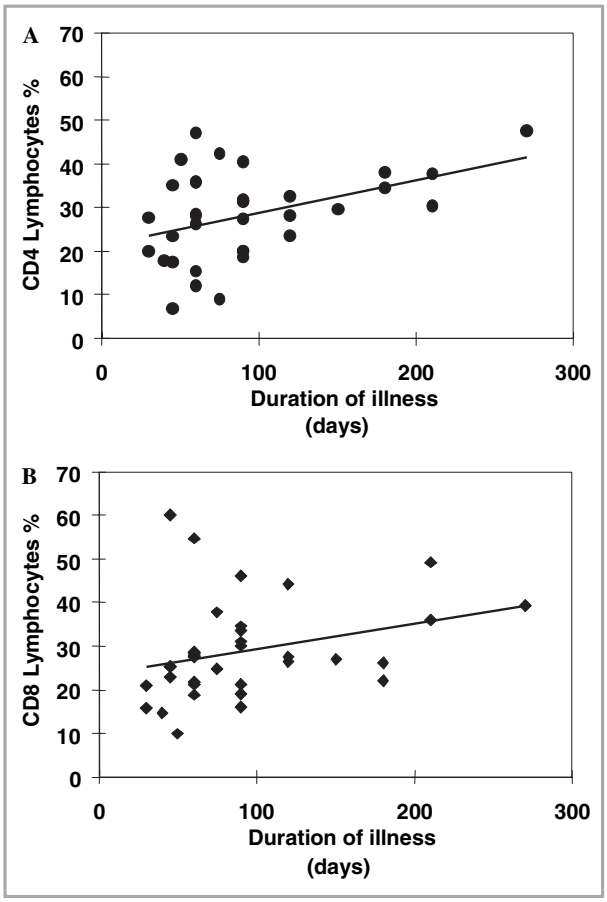

Fig 1. Relationship between the proportions of CD4+ (A) and CD8+ (B) T-cell subsets and the duration of illness of cutaneous leishmaniasis lesions.

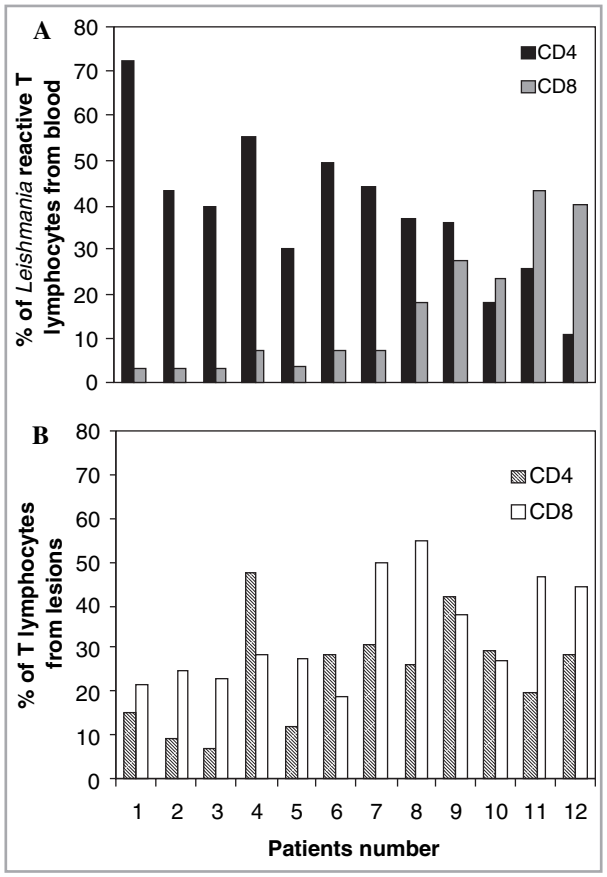

Fig 2. Comparison between the proportions of *Leishmania*-reactive CD4+ and CD8+ T cells in peripheral blood (A) and lesions (B) from 12 cutaneous leishmaniasis patients.

subsets (Fig. 2A) and CD4/CD8 ratios in the inflammatory infiltrate (Fig. 2B).

Discussion

Infection by *Leishmania* parasites induces a chronic granulomatous inflammatory disease leading to an increase of lymphocytes, plasmocytes and macrophages in skin. Although scanty in normal skin,[1] lymphocytes were the predominant mononuclear cell population in leishmaniasis lesions, as demonstrated previously.[2,18,20–23] The predominance of T cells was more remarkable in ML than in CL or DissL. A higher frequency of *Leishmania*-responding lymphocytes is also observed in mucosal lesions,[13] suggesting that these cells could be part of the hypersensivity reaction to parasite antigens observed in ML patients.

The number of cells expressing IL-2 and IFN-γ receptor was also higher in ML than in CL patients, indicating the level of T-cell activation in response to *Leishmania* antigenic stimulation.[18,21,29] In addition, experimental data have shown that resistant mice lacking IFN-γR become susceptible to *L. major* infection, even in the presence of a type 1 response.[32] In humans, although IFN-γ mRNA-containing cells were present in similar percentages in both CL and ML lesions,[6] this cytokine is produced in higher amounts by ML than CL patients.[10,12] Therefore, the higher expression of IFN-γR in mucosal cells could render ML patients more susceptible to a possible harmful effect of an excess of IFN-γ. Taken together, these data strengthen the idea that T cells and cytokines, although fundamental to the control of parasite infection, could also play an important role in mucosal tissue damage.

In lesions caused by *L. braziliensis*, a predominance of CD4+,[18,20] CD8+,[22,23] and equivalent proportions of these T-cells subsets[21] have been described. In patients studied here, the overall percentages of CD4+ and CD8+ T cells were similar in CL and ML lesions, although the proportion of these cells was extremely variable among individuals suffering from the same clinical form. Beyond this, the CD4/CD8 ratio was not correlated with the number or location of lesions, MST intensity or histopathological findings (granuloma, necrosis or vasculitis) (data not shown). The heterogeneity of T-cell subpopulation patterns was also demonstrated by immunocytochemical studies in cutaneous lesions caused by *L. panamensis*.[29] This feature could account for the controversial data available regarding the CD4+ and CD8+ T-cell composition of inflammatory infiltrate.[18,20–23] Despite this, it is unexpected that

there is no correlation between CD4+ or CD8+ infiltrating T cells and the pathogenesis of human leishmaniasis lesion.

It is known that histopathological findings from leishmaniasis lesions include a transitory but reversible cellular pattern, which can be modified according to the disease progression.[17] Distinct histopathological patterns are also observed in the same lesion depending on the site where the tissue is taken from.[19] Similarly, granuloma organization has a remodelling pattern that comprises a duality of synthesis and degradation of extracellular matrix, combining in the same lesion an acute inflammatory infiltrate together with a significant reparative process.[33] These factors could mirror a constant host effort to heal the disease, which is compatible with the self-healing profile of cutaneous leishmaniasis. It could be hypothesized that the composition of the inflammatory cells could be reflecting distinct phases of the outcome of the lesion, which could explain the variability of results observed not only in our flow cytometry studies but also in immunohistochemical analyses performed by different authors.

The patients described here showed an increase in proportion of CD4+ and CD8+ T cells in lesions older than 3 months (Fig. 2). The organization of the inflammatory infiltrate seems to be a dynamic process possibly influenced by the development of the immune response.[34] The persistence of inflammatory response could favour the increase in lymphocytes, which may result from both continued recruitment of leucocytes, *in situ* proliferation and/or lack of clearance of lesion-infiltrating cells.[35] A higher frequency of cell-death events in CD8+ T cells observed in CL lesions was associated with the active form of the disease. In addition, low frequencies of early apoptotic events among CD8+ T cells were observed in self-healing lesions.[36] Therefore, differences in apoptotic events in CD4+ and CD8+ T-cell subsets could be responsible for controlling the CD4/CD8 ratio, thus leading to healing or maintenance of disease. In accordance with this fact, it has been suggested that dysregulation of T-cell apoptosis may contribute to disease chronicity via inability to terminate the ongoing response.[35] The increase in T lymphocytes in lesions could reflect a regulatory failure of T-cell subsets in inflammatory infiltrates, which is probably associated with the poor outcome of the disease.

γδ lymphocytes were present in variable amounts in CL, ML and DissL lesions, independent of the clinical characteristics of lesions including duration. Although γδ cells have been associated with the initial phases of granuloma formation,[21,37] our data allow us to argue that if γδ cells participate in antigenic recognition or cytokine production, this occurs whatever the clinical form or chronicity level.

B cells were present in the cellular infiltrates of CL and DissL in similar levels, differing in this aspect from other studies.[22] Although B cells are not present in normal skin,[1] they have been demonstrated in leishmaniasis lesions.[17,20–22,24,29] Influx of these cells into lesions and immune complex deposition could also be involved in the pathogenesis of leishmaniasis lesions,[38] as high levels of antibodies are detected in more severe clinical forms.[39] However, the exact role of these cells in the outcome of leishmaniasis is not known.

The proportions of intralesional CD4+ and CD8+ cells had no association with T-cell subsets in PBMC or even with *Leishmania*-reactive T cells derived from blood. It would not be expected that immune responses in lesions would mirror peripheral T-cell immunity or even that lesions functioned as a filter of blood.[2] However, the low percentages of *Leishmania*-reactive CD8+ T cells in peripheral blood could indicate that those cells are migrating to the lesions. Enrichment of CD8+ cells was observed in the inflammatory infiltrate, as demonstrated previously,[20] suggesting that local immunoregulatory factors could favour the recruitment and/or proliferation of those cells. This observation, associated with the time-related increase of these cells in combination with lower numbers of parasites found in older lesions, points to the possible cytotoxic role of these lymphocytes in the control of parasite replication. However, the possible role of these cells in tissue damage or even chronicity of lesions cannot be ruled out.[7,9,10,40,41]

Specific primed T cells express molecules that mediate homing to the lesions.[42] Although *Leishmania*-reactive T cells are present in the lesions, they probably do not constitute all the T-memory lymphocytes observed in the infiltrate.[13,18,20] Data from our laboratory demonstrated that T lymphocytes derived from lesions of ATL patients can also react against *Toxoplasma gondii* (Tg) antigens, suggesting that lymphocytes other than *Leishmania*-specific T cells can migrate to the inflammatory infiltrate of leishmaniasis lesions (manuscript in preparation).

Finally, flow cytometry can be used as an approach to address the immunopathogenic mechanisms occurring at the site of infection. Moreover, results from flow cytometry are representative of the whole lesion sample, whereas *in situ* immunohistochemical studies analyse a particular microscopic field. Our results demonstrated that despite significant variability in the frequency of intralesional T-cell subsets, increased proportions of intralesional CD4+ and CD8+ T cells were observed in lesions of long duration. Indeed, an enrichment of CD8+ T cells in lesions is consistent with the hypothesis that they can mediate healing. On the other hand, it is not possible to exclude the involvement of these cells in the chronicity of lesions due to the absence of parameters to predict the clinical course of lesions. Determination of the specificity of these T cells and characterization of their effector functions constitute important steps in understanding the immune mechanisms involved in the pathogenesis of leishmaniasis. It is possible that these mechanisms can influence the clinical outcome or even the response to therapy.

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