Short Communication

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Immunophenotypic characterization of patients with American cutaneous leishmaniasis prior to and after treatment in Pernambuco, Brazil

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Abstract: *Leishmania* infections induce a specific activation of host immunological response, particularly characterized by T cell expansion. Studies indicate the importance of the balance between $CD4^+$ and $CD8^+$ T cells, in which the first ones would have their number reduced during the healing process. Meanwhile, $CD25^+$ T cells have been associated with the suppression of the immune response. Since the immune response has an essential role in both healing and progression of diseases, this study aimed to identify the percentage of $CD3^+$, $CD4^+$, $CD8^+$, $CD16^+$ and $CD25^+$ T cells in the peripheral blood of patients afflicted with American cutaneous leishmaniasis (ACL) – before and after treatment – and healthy controls. Peripheral blood was collected and transferred to cytometry tubes containing monoclonal antibodies specific for cell surface markers CD3, CD4, CD8, CD16 e CD25. The immunophenotypic and morphometric parameters of cells were determined by flow cytometry and the results demonstrated a significant increase in the number of T $CD8^+$ cells after treatment, suggesting a cytotoxic T cell response. An increase in $CD25^+$ T cells in patients with active ACL and after treatment was also observed, suggesting the participation of these cells in the modulation of the exacerbated effector response.

Key words: American tegumentary leishmaniasis, cellular immune response, flow cytometry, *Leishmania (Viannia) braziliensis*.

American cutaneous leishmaniasis (ACL) is an anthropozoonotic disease caused by protozoans of the genus *Leishmania* and transmitted to humans by the bite of *Lutzomyia* phlebotomines (1-5). *Leishmania (Viannia) braziliensis* is the causative agent of most cases of ACL in Brazil, especially Pernambuco (5-7). Clinical manifestations vary from localized cutaneous ulcers to mutilating mucocutaneous lesions, and depend on parasite species, vector, epidemiological characteristics, genetic constitution, and immunological conditions of the host (4, 7, 8).

Infections by *Leishmania* lead to a specific activation of the host immune response (9). Studies indicate the importance of the balance between T $CD4^+$ and $CD8^+$ cells, in which the first ones would have their proportion reduced during the healing process (10, 11). Meanwhile, natural killer (NK) cells participate by secreting IFN-γ, important for the differentiation of Th1 and Th2 responses by $CD4^+$ T cells, and also through direct cytotoxic effect on parasitized macrophages (12). In the meantime, $CD25^+$ T cells have been associated with the suppression of the immune response by effector T cells (13, 14).

In the present study, the proportions of $CD3^+$, $CD4^+$, $CD8^+$, $CD16^+$ and $CD25^+$ T cells in peripheral blood of patients with ACL prior to and after treatment were analyzed and compared with healthy individuals. Blood samples were obtained from ten patients with active ulcers and, then, six months after the end of therapy samples

were collected from seven of them, and also from ten healthy controls living in non-endemic areas.

Ten patients with localized cutaneous lesions were studied. The following criteria were used for diagnosis: type of lesion and epidemiological data compatible with cutaneous leishmaniasis (CL); positive Montenegro skin test (MST); detection of *Leishmania* parasites in skin lesions by PCR; presence of *Leishmania*-specific immunoglobulin G antibodies in serum samples detected by indirect immunofluorescence.

All the patients came from areas in which leishmaniasis is endemic in the state of Pernambuco, Brazil. They were treated with Glucantime® (Sanofi-Aventis, USA) and were considered cured six months after their lesions had completely healed. All ten patients (five men and five women) had active leishmanial skin ulcers at the beginning of the study (Table 1).

The mean age of the patients was 25.25 ± 9 years. Seven patients had a single lesion and three had two to four lesions. MST was positive for all patients, with areas of induration ranging from 8 to 16 mm in diameter. The detection of *Leishmania* parasites by PCR was performed in six of the ten studied patients and it was positive for all of them. Immunofluorescence test was performed on samples from all patients and tested positive in 70% of them. In patients who tested negative by immunofluorescence, the detection of *Leishmania* parasites was accomplished by PCR and immune response through MST, which resulted positive in all of them, thus, confirming the diagnosis.

The percentages of studied cell populations were determined for all patients as the proportion of gated peripheral blood cells in the lymphocyte region, through flow cytometry graphs. Peripheral blood was collected in tubes containing EDTA and transferred to cytometry tubes containing monoclonal antibodies anti-CD3-Fitc (Miltenyi Biotec, USA), anti-CD4-Fitc (eBioscience, San Diego, CA, USA), anti-CD8-PE (eBioscience, USA), anti-CD16-PE (Caltag, USA) and anti-CD25-APC (BD Pharmingen, San Jose, CA, USA). After homogenization in a vortex, the mixture was incubated for 40 minutes. Following that, the samples were submitted to erythrocyte lysis, followed by washes and centrifugations, with posterior resuspension in 400 μL of 1% paraformaldehyde. The immunophenotypic and morphometric parameters of cells were determined by flow cytometry.

The results indicated a similar profile between

Table 1. Clinical, epidemiological and laboratory aspects of patients with American cutaneous leishmaniasis in the state of Pernambuco, Brazil

Patient	Age	Gender	Area	Lesions	Lesion type	MST	PCR	IFI
J. S. A	32	M	Moreno	2	Necrotic ulcer	8 mm	+	+
J. A. S	26	M	Moreno	1	Necrotic ulcer	10 mm	NI	+
C. D. S	20	M	Moreno	1	Necrotic ulcer	10 mm	NI	+
M. S. B.	17	F	Moreno	1	Necrotic ulcer	8 mm	NI	+
V. M. P. L.	26	F	Moreno	1	Necrotic ulcer	15 mm	+	+
D. A. S.	15	F	Moreno	4	Necrotic ulcer	12 mm	+	+
R. S. A.	43	F	Jaboatão dos Guararapes	2	Necrotic ulcer	16 mm	+	–
A. M. O	23	M	Recife	1	Necrotic ulcer	14 mm	+	–
M. J. P. F.	NI	F	Moreno	1	Necrotic ulcer	9 mm	+	–
M. F. L.	NI	M	Moreno	1	Necrotic ulcer	14 mm	NI	+

MST: Montenegro skin test; PCR: detection of the parasites by polymerase chain reaction; IFI: immunoglobulin detection by indirect immunofluorescence; +: positive; –: negative; NI: not informed.

controls and patients prior and after treatment concerning CD3-, CD4- and CD16-positive populations (Table 2). Similar values were obtained by Autissier *et al.* (15) and Faria *et al.* (16) when evaluating healthy volunteers. Botelho *et al.* (12), studying the influence of treatment in phenotypic profile of patients, also did not notice significant differences in the $CD3^+$, $CD4^+$ and $CD16^+$ cells, between patients and controls. These results suggest that no harmful changes were induced by the treatment.

It was observed a significant increase in T $CD8^+$ proportion in patients after treatment in comparison to controls ($p = 0.0250$) and patients with active lesions ($p = 0.0418$) (Table 2, Figure 1).

That reflected in the $CD4^+/CD8^+$ ratio (2.79 ± 0.83 in controls; 3.22 ± 1.18 in patients with active lesions; 2.34 ± 0.96 in patients after treatment), which decreased in these patients, as shown in Figure 2. It is known that $CD8^+$ T cells participate in the immune response by a cytolytic effect upon parasitized macrophages. $CD8^+$ T cells have been associated with protection against *Leishmania* reinfection in murine models; however, the induction of these T-cell subsets in humans seems to be also related to the healing process (17). $CD8^+$ T cells increased proportion after treatment suggests that the cytotoxic effect of these cells on parasitized cells may contribute to the elimination of the parasite.

Regarding the proportion of $CD25^+$ T cells, a two-fold increase was observed between patients prior to treatment and controls ($p = 0.0068$) and also between patients before and after treatment ($p = 0.0010$); a four-fold increase between patients after treatment and controls ($p = 0.0002$) was also observed (Table 2, Figure 3). Belkaid *et al.* (14) explain that these cells regulate the function of local effector cells, which prevents efficient elimination of the parasite. In this model of response, parasite persistence, as a result of

Table 2. Proportion of T cells ($CD3^+$), T helper cells ($CD4^+$), cytotoxic T cells ($CD8^+$), natural killer cells ($CD16^+$) and regulatory T cells ($CD25^+$) in controls (CT), patients with active lesion (AT) and patients after chemotherapy (PT)

	CT	AT	PT
CD3	64.12 ±5.41	61.38 ±3.91	63.13 ±5.7
CD4	38.17 ±4.78	39.75 ±1.8	42.09 ±5.9
CD8	14.70 ±4.33	13.35 ±4.27	19.58 ±5.36#
CD16	11.92 ±6.11	12.02 ±3.72	10.89 ±6.21
CD25	3.22 ±1.6	6.42 ±2.63#	12.91 ±2.82#

# $p < 0.05$

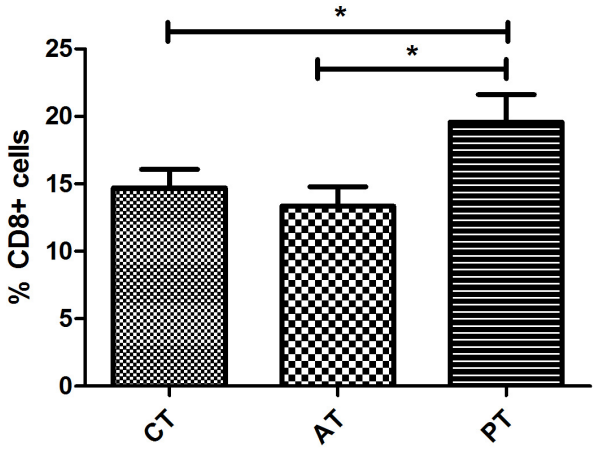

Figure 1. Proportion of cytotoxic T cells (CD8+) in the peripheral blood of controls (CT), patients with active lesion (AT), and patients after chemotherapy (PT). Differences between groups were considered significant when $p < 0.05$ and are indicated by asterisks (*).

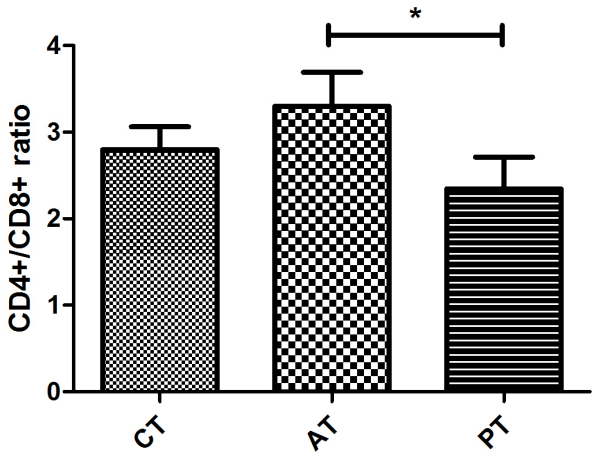

Figure 2. $CD4^+/CD8^+$ ratio in the peripheral blood of controls (CT), patients with active lesion (AT), and patients after chemotherapy (PT). Differences between groups were considered significant when $p < 0.05$ and are indicated by asterisks (*).

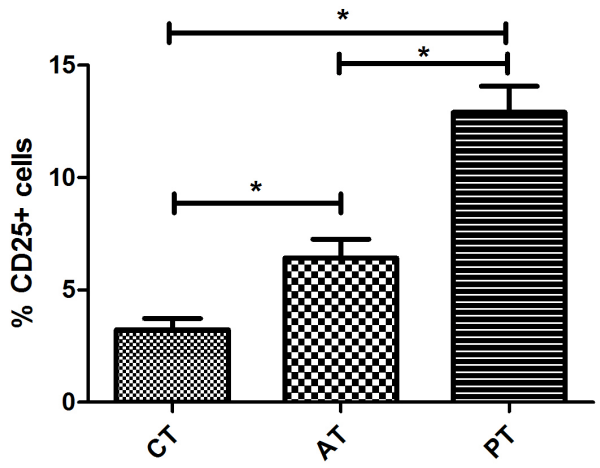

Figure 3. Proportion of regulatory T cells ($CD25^+$) in the peripheral blood of controls (CT), patients with active lesion (AT), and patients after chemotherapy (PT). Differences between groups were considered significant when $p < 0.05$ and are indicated by asterisks (*).

immune suppression by regulatory T cells, is necessary for the maintenance of protective immunity against the parasite.

After successful immunologic (or chemotherapeutic) control of *Leishmania* infection, a small number of parasites persisted, a situation that is associated with immunity to reinfection (known as "concomitant immunity"). Therefore, we believe that the immune response plays a fundamental role in the development of leishmaniasis and that $CD8^+$ T cells are associated with the healing process. Furthermore, we consider that the $CD25^+$ T cell expansion, presented by patients, may be due the role of these cells in the modulation of an exacerbated response by effector T cells, and maintenance of a small number of parasites in the localized lesion as an antigenic stimulus to prevent reinfection.

Additional studies with more patients and markers are under development for a better comprehension of this process dynamics.

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CONFLICTS OF INTEREST

There is no conflict.

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ETHICS COMMITTEE APPROVAL

The present study was approved by the Aggeu Magalhães Research Center Ethics Committee (CAEE 0757.0.095.000-05). In addition, all studied subjects signed an informed consent form.

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