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American cutaneous leishmaniasis: In situ immune response of patients with recent and late lesions

A. H. S. Gomes[1] | R. B. Martines[2] | C. T. Kanamura[2] | M. L. P. Barbo[3] | S. D. Iglezias[2] | J. A. Lauletta Lindoso[4,5,6] | V. L. Pereira-Chioccola[7]

[1]Laboratorio Regional de Sorocaba, Instituto Adolfo Lutz, Sao Paulo, Brazil

[2]Centro de Patologia, Instituto Adolfo Lutz, Sao Paulo, Brazil

[3]Departamento de Morfologia e Patologia, Faculdade de Ciências Médicas e Saúde, Pontifícia Universidade Católica, Sorocaba, São Paulo, Brazil

[4]Instituto de Infectologia Emilio Ribas, São Paulo, São Paulo, Brazil

[5]Laboratório de Soroepidemiologia (LIM-38) Hospital das Clínicas da Faculdade de Medicina da, Universidade de São Paulo, São Paulo, Brazil

[6]Instituto de Medicina Tropical de São Paulo, Universidade de São Paulo, São Paulo, Brazil

[7]Centro de Parasitologia e Micologia, Instituto Adolfo Lutz, Sao Paulo, Brazil

Correspondence
Vera Lucia Pereira-Chioccola, Centro de Parasitologia e Micologia, Instituto Adolfo Lutz, São Paulo, SP, Brazil.
Email: pchioccola@gmail.com

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Summary

TNF-α, IFN-γ, IL-10, IL-17, CD68 and CD57 were evaluated in biopsies of patients with American cutaneous leishmaniasis living in Sorocaba, Brazil. The analyses were performed considering the time of lesions from 23 patients with recent lesions (Group I) and 19 patients with late lesions (Group II). All patients were infected with *Leishmania (Viannia) braziliensis*. Immunostaining cells for CD68, CD57, TNF- α, IFN-γ, IL-10 and IL-17 were performed by immunohistochemistry. Except for CD68 and IL-17, the distribution of in situ for CD57, IL-10, TNF-α and IFN-γ showed that patients with recent lesions expressed higher levels than those with late lesions. The comparison of cytokine expression/group showed that IL-10 was significantly higher than IL-17 and IFN-γ (similar data were shown in IL-17 compared with TNF-α), suggesting an immunological balance between inflammatory-anti-inflammatory agents. This balance was similar for two groups of patients. In conclusion, these data suggested that (i) patients from Group I had recent lesions (in the beginning of chronic phase) compared to those from Group II and (ii) the modulation of inflammatory response in patients with recent American cutaneous leishmaniasis was correlated with IL-10 expression in skin lesions preventing the development of mucosal forms. The parasite treatment also prevented the evolution of severe forms.

KEYWORDS
American cutaneous leishmaniasis, *Leishmania (Viannia) braziliensis*, pro-inflammatory and anti-inflammatory cytokines

1 | INTRODUCTION

The genus *Leishmania* causes leishmaniasis, which comprises a group of chronic diseases transmitted from animals to humans by the bite of infected female sand flies. There is a broad spectrum of clinical forms, including those that affect skin, mucosa or internal organs.[1,2]

The prevalence of the cutaneous forms caused by *Leishmania* is estimated to be between 0.7 million and 1.3 million new cases worldwide annually.[3] American cutaneous leishmaniasis (ACL) is widely distributed in the Americas, from northern Argentina to southern USA. From 2001 to 2011, around 270 500 cases were reported, with an average of 27 500 new cases/year.[4] In Sao Paulo State (Brazil), the incidence is approximately 400 new cases per year.[5]

American cutaneous leishmaniasis can be caused by a protozoon belonging to the *Leishmania (Viannia) braziliensis*. Infected patients can have spontaneous cure or develop cutaneous lesions. However, the localized cutaneous form is the most prevalent, with about 90% of cases.[6]

The immunological status and clinical evolution in ACL are very closely related. The cellular immune response is crucial to controlling the infection, but it is also harmful to tissues leading to destructive lesions, and the production of cytokines is one the most important events in parasite evolution in human infection because it is involved

in the lesion development, cure evolution or worsening relapsing forms.[7,8]

Usually, the initial lesions begin at the site of the sand fly bite and progress to a nodule and ulceration after 1-3 months. Depending on the geographic region and the *Leishmania* species involved, cutaneous lesions are classified as acute or chronic.[1,7,8] However, the correlation between time of the lesions and evolution is poorly known and controversial. Douba et al. [9] analysing 1750 patients from Aleppo (Syria) with cutaneous lesions caused by *L. (L.) tropica* suggested that lesions with <1 year of evolution could be classified as an acute form and the chronic ones being those persistent for more than 1 year. Costa-Silva et al. [10] classified ACL caused by *L. (V.) braziliensis* as acute in lesions until 3 months and chronic ones after 3 months of evolution. Certainly, the *Leishmania* species and the time of evolution of lesion can influence the immune response in situ.

CD4 T cells are considered the most important subpopulation of cells involved in ACL evolution. CD8 T cells are also important, but in the initial stages of infection-producing interferon γ (IFN-γ). However, cytotoxic CD8 T cells are harmful to both *L. (V.) braziliensis* and infected host cells, because cytotoxicity is higher in mucocutaneous leishmaniasis than the cutaneous form.[11,12] Other cells, particularly CD4+ CD25+ (T regulatory cells, Treg cells), accumulate in cutaneous lesions caused by *L. (V.) braziliensis* contributing to control of effector T-cell functions.[13] Valencia-Pacheco et al.[14] analysing in situ gene expression of some cytokines and chemokines in active and healed lesions caused by *L.(L.) mexicana* observed predominance of IL-12 and MCP-1 and absence of IFN-γ gene expression in healed lesions, suggesting that this molecule could play a role in early infection. Additionally, different cells contribute to the production of interleukin-10 (IL-10), including macrophages during *Leishmania* infection. IL-10 plays an important role in downregulating the Th1 immune response and facilitates parasite growth, and it may play an important role in the initial phase of the infection.[15,16]

In chronic lesions, the cellular infiltrates are characterized mainly by B and T cells.[7,11] The infectivity of some species of *Leishmania* is partly due to their ability to repress the induction of pro-inflammatory cytokines, making their entry less detectable to the host.[8,12,17–19] The course of the infection is characterized by strong cellular responses and rare parasites in lesions.[8] The presence of activating cytokines, such as IFN-γ and tumour necrosis factor alpha (TNF-α), is decisive for controlling parasite dissemination, but an exaggerated Th1 response has been associated with the severe inflammation observed in ACL lesions.[17,18,20]

Despite substantial progress in *Leishmania* immunobiology, the overall of immunological mechanism has not yet been fully clarified. Different studies evaluated the immune response in late forms of infection such as mucosal, reoccurrences and more severe disorders caused by *L. (V.) braziliensis*.[12–14,21–25] In addition, the role of Th17 and its correlation with Th1/Th2 responses were not totally established in the beginning of the chronic infection by *L. (V.) braziliensis*.

Different studies have shown the effect of the time evolution of lesions in the immunological profile of patients with ACL.[11,24,26] According to Costa-Silva et al.,[10] in lesions caused by *L. (V.) braziliensis*, 3 months is an appropriate time to classify recent and late lesions, because the gene expression profile of cytokines and chemokines of patients with recent lesions is distinct from those with late lesions. In addition, the time evolution of lesions can interfere with the sensitivity of different laboratorial methods.[27]

All these data lead us to study patient immunological status because it can contribute to prompt diagnosis and specific treatment, to prevent disfigurations and psychological disorders. Thus, this study evaluated the localized immune response in patients attending Public Dermatologic Clinics in Sorocaba, an endemic region, in Sao Paulo State, Brazil. This region is considered the second most endemic region for cutaneous leishmaniasis in Sao Paulo State (about 90 new cases per year),[5] comprising 45 cities (small to medium size) with rural, periurban and urban localities.

The analyses were performed in lesion biopsies to determine the immune expression of different cytokines as well as, CD68 and CD57 cells, considering the time evolution. Patients were classified in two groups according to Costa-Silva et al.[10] The results suggest that variations in expression of some cytokines could be important for infection evolution.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

The procedures were performed after patient's signed informed consent forms. All patients with skin lesions and individuals from the control group accepted, approved and signed the documents. The Ethic Committee of the Instituto Adolfo Lutz (Sao Paulo, Brazil) approved the patient's written informed consent and the entire study. The study was performed according to recommendations of the Human Ethics Committee (CONEP-IAL number: 424.827).

2.2 | Patients and biopsy samples

This study was conducted analysing 112 skin biopsies collected for 14 months (June 2012 to July 2013) from patients with cutaneous lesions living and attending public dermatology clinics of Sorocaba Region, Sao Paulo State, Brazil. As Sorocaba is a high endemic region for ACL, all patients who presented typical cutaneous lesions suggestive of leishmaniasis were given a complete dermatological examination performed before skin biopsy collection. The medical procedures were performed according to recommendations of the Brazilian Health Ministry as previously described in a technical manual.[5,27] None of them had acquired the infection before or had been treated with drugs for leishmaniasis. The lesions were cleaned with an antiseptic, and a local anaesthetic was administered. The borders of the lesion were scraped and samples were obtained by punch biopsy (0.2 mm) and immediately added to three tubes. Two tubes contained 1-2 mL of sterile 0.85% NaCl and 200 μg/mL gentamicin solution and were processed for parasitological test (microscopic examination) and DNA extraction. The third tube contained 1-2 mL of sterile 0.85% NaCl and 10% formalin and was processed for histopathological diagnosis and immunohistochemistry (IHC) analysis. The samples were sent to the laboratory within 48 hours, where they were immediately processed. A control

group (Group III) was composed of 14 samples of normal skin tissues collected from females who had undergone cosmetic surgery. The skin tissue samples were collected, and samples were obtained and immediately added in a tube contained 1-2 mL of sterile 0.85% NaCl and 10% formalin for histopathological, IHC and molecular analysis.

2.3 | Histopathological, parasitological and molecular diagnosis

For histopathological analyses, skin tissue samples were fixed in buffered formalin. Fixed fragments were embedded in paraffin and stained with haematoxylin-eosin (H&E) after microtome sectioning. Histologic sections were examined under light microscope to determine tissue characteristics.

For parasitological diagnosis, skin biopsy imprints were plated onto a glass slide, fixed with methanol and stained with Giemsa. The presence of amastigotes was observed microscopically with an immersion objective (×1000).[27,28]

Before performing the DNA extraction, biopsy samples were crushed and digested, in a lysis buffer (Tris-HCl, 10 mmol/L, pH 8.0; EDTA 10 mmol/L; SDS, 0.5%; N-laurilsarcozil, 0.01%; proteinase K, 100 μg/mL), briefly vortex mixed and incubated at 56°C until complete cell lysis (5-24 hours).[27] Then, the DNA was automatically extracted by QIAamp DNA Mini Kit®(Qiagen, GmbH, Hiden, Germany), according to the manufacturer's instructions in the QIAcube® instrument (Qiagen). DNA concentrations and purity were determined by the ratio of O. D. at 260 and 280 nm in a NanoDrop ND1000® (Thermo Fisher Scientific, Waltham, MA, USA). *Leishmania* genus was identified by PCR designed to amplify a 120-bp fragment from a conserved region of *Leishmania* spp. kDNA minicircles using primers 150/152.[29] *Leishmania (V.) braziliensis* were determined by amplification of a fragment ranging from 146 to 149 bp, from the multicopy spliced leader RNA gene using the primers LU-5A/LB-3C.[28] The reactions were carried out with Go Taq Green Master Mix® (Promega, Madison, Wisconsin, EUA) containing 1 mmol/L of each primer and 5 μL of DNA in a final volume of 25 μL. To verify the quality of the extracted DNA and presence of PCR inhibitors, all samples were assayed by a PCR that amplifies a housekeeping gene that amplified a 140-bp fragment of the human β-globin gene using primers β1/β2 following the same conditions as previously described.[28] After thermal cycles, PCR products were electrophoresed in 2% agarose gel and stained with ethidium bromide. DNA fragments were made visible by UV illumination, and images were analysed by a Syngene GeneSnap Imager® (Frederick, MD, USA), version 6.05.01. The sizes of fragments were based on comparison with a 100-bp ladder.

2.4 | IHC analysis in formalin-fixed paraffin-embedded (FFPE)

To assess the aetiologic agents, inflammatory cells and cytokine distribution in skin lesion, IHC analysis was performed on deparaffinized FFPE sections (4 μm thick) using monoclonal or polyclonal antibodies. Positive and negative controls consisted of human lymph node tissue sample and the primary antibody omission, respectively.

Immunostained procedures were performed after removing paraffin in xylene and rehydrating in baths with decreasing concentrations of ethanol and in distilled water and then submitted to antigen retrieval procedure in 10-mmol/L citrate buffer pH 6.0. Endogenous peroxidase activity blockage was obtained with 6% peroxide hydrogen solution treatment.

For leishmaniasis diagnosis, the sections were immediately incubated with a mouse polyclonal anti-*Leishmania* spp. Inflammatory cell and cytokine distribution in skin lesions were performed using rabbit polyclonal anti-IL-10 (Abcam, USA, diluted 1:1 000 000), rabbit polyclonal anti-IL-17 (Abcam, San Francisco, diluted 1:1000); mouse monoclonal anti-TNF-α (P/T2, Abcam, diluted 1:1000); rabbit polyclonal anti- IFN-γ (Abcam, diluted 1:5000), mouse monoclonal anti-CD57 (NK cells) (NK-1, Novocastra, UK, diluted 1:400), mouse monoclonal anti-CD68 (KP1; Dako, USA, diluted 1:4000). All antibodies were diluted in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), and tissue sections were incubated overnight at 4°C. Amplification signal was obtained by peroxidase conjugated polymer (Spring Biosciences, CA, USA), in a 30-minute incubation step at 37°C. Colour development was obtained with diaminobenzidine (Sigma, Sigma-Aldrich, USA, 100 mg% in PBS, 0.1% peroxide hydrogen) chromogenic substrate, followed by light counterstaining with Harry's haematoxylin and permanent mounting with Entellan (Merck, Germany).

2.5 | Data analysis

Clinical and epidemiological data as well as laboratory criteria were used to establish the ACL diagnosis. According to the laboratorial diagnosis, samples were considered positive when positive results were shown in at least two laboratory procedures.

The quantitative morphometric observations were made by manually counting the number of immunostaining cells in ten high-powered, fields/biopsy, light microscopes (400× magnification). The number of positive cells was statistically analysed using an unequal-variance *t* test based on a critical value of $P \leq .05$ and F test to compare variances. All analyses were performed using GraphPad Prism 6.0 Software Inc. (San Diego, CA, USA).

The clinical, epidemiological and laboratorial data were used to identify the patients with ACL. Next, they were divided into two groups based time of appearance lesion. Group I was composed of patients whose lesions appeared within 3 months (recent infection). Group II was composed of patients whose lesions appeared more than 3 months after infection (late infection). This classification was based on previous studies. Three months is the correct time period for classifying recent and late lesions in ACL.[10] All of lesions were considered in the chronic phase of the infection, since in early stage or pre-ulcerative phase, the lesions are not present.[29,30]

3 | RESULTS

3.1 | Patients and diagnosis

During the study period (14 months), the 112 patients clinically suspected of having ACL referred to the public dermatology clinics were

diagnosed by clinical, epidemiological and laboratorial data. Therefore, in Sorocaba an endemic region for ACL, it is common that patients have other chronic ulcers. Of the 112 samples, 70 biopsies were negative for *Leishmania* (in the four laboratory procedures). Among these patients, the histopathological diagnosis was positive for vasculopathic ulcers, skin carcinomas (basal and squamous cell carcinoma) or fungal diseases (paracoccidioidomycosis and sporotrichosis). The other 42 (37.5%) patients were defined as having ACL. Parasites were identified by, at least two of the methods employed. A detailed distribution of the laboratorial-epidemiological features of the patients with ACL is shown in Table 1. Patients were divided according to the lesion duration (in months). The recent lesions ranged from 1 to 3 months in 23 patients (Group I). The later lesions were shown in 19 patients and ranged from 4 to 24 months (Group II). Patients were divided into 18 males and five females (Group I) and 10 males and nine females (Group II). The ages ranged from 11 to 74 years in both groups.

A single lesion was seen in the majority of the cases (37/42, 88%) and was mainly located on the legs/feet (20 cases, 48%) and arms/hand (eight cases, 19%). Multiple lesions were shown only in five (12%) patients with later lesions. No statistical difference was observed between groups I and II according to the distribution of the lesions.

According to the laboratorial diagnosis, the 42 positive patients had the following results. Parasites were detected by the parasitological method in 64% of the patients (27/42), whereas 100% of them (42/42) had positive results in PCR. The DNA samples were tested by PCR to determine *Leishmania* ssp. and for *L. (V.) braziliensis*. Positive results for both primer sets confirmed that all positive patients analysed in this study were infected with *L. (V.) braziliensis*. The control of the PCR inhibitors by the positive results for human β-globin confirmed the good quality of the DNA extracted from the biopsies. Positive IHC for *Leishmania* were shown in 95% of the patients (40/42). Individuals from Group III (control) were 14 females ranging from age of 35-54 years. All samples were negative for leishmaniasis in all laboratorial reactions.

The biopsy preparations were used to identify amastigotes, and the histopathological results showed different epidermal changes such as hyperkeratosis, acanthosis, pseudoepitheliomatous hyperplasia (Figure 1A) or ulceration and intra-epidermal microabscesses. Dermis presented inflammatory infiltrate with lymphocytes, plasma cells,

TABLE 1 Characteristics and distribution of clinical, laboratory and epidemiological features from the 42 patients with American cutaneous leishmaniasis (ACL) and the 14 individuals from the normal control

Patient features n=42	Group I	Group II	Total patients with ACL	Group III n=14
Lesion duration (in months)				
1-3	23	-	23	-
4-24	-	19	19	-
Gender				
Male	18	10	28	0
Female	05	09	14	14
Age (years)				
≤15	03	03	06	-
15-34	05	07	13	-
35-54	11	07	17	14
55-64	03	01	04	-
≥65	01	01	02	-
Lesion				
Legs/feet	10	10	20	0
Arms/hands	07	01	08	0
Chest	02	02	04	0
Head	02	03	05	0
Multiple lesions	02	03	05	0
Parasitological Method				
Positive	14	13	27	0
Negative	09	06	15	14
PCR: Leishmania ssp. and *Leishmania (Viannia) braziliensis*				
Positive	23	19	42	0
Negative	0	0	0	14
Immunohistochemistry and histopathology				
Positive	21	19	40	0
Negative	02	0	02	14

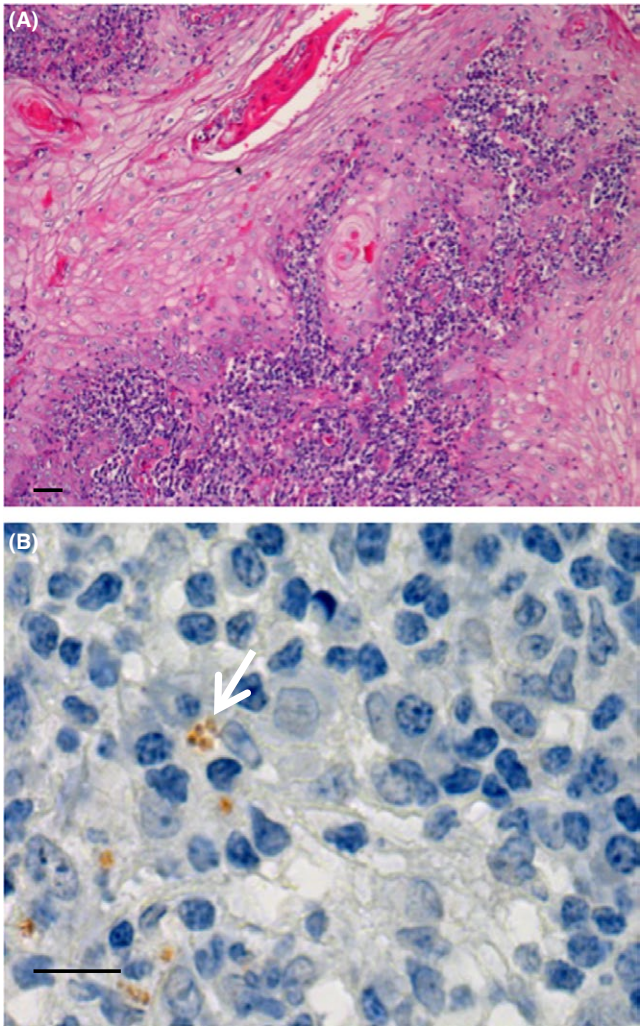

FIGURE 1 Cutaneous histopathological features and IHC in biopsy of American cutaneous leishmaniasis (ACL). (A) Pseudoepitheliomatous epidermal hyperplasia with a diffuse inflammatory response. (B) *Leishmania* amastigotes (arrows) are highlighted by IHC staining with a mouse polyclonal anti-*Leishmania* spp, as described in Material and Methods section. Original magnifications: ×10 (A) and ×100 (B). Scale bar 20 μm (A) and 10 μm (B)

macrophages containing amastigotes and rare eosinophils. Epithelioid and giant cells with granulomas were observed in only 16.6% of cases of Group I (lesions until 3 months). Mild intracellular and free amastigotes were visible on H&E and IHC staining in the majority (40/42) of the samples (Figure 1B).

3.2 | *In situ* cytokine expression

Figure 2 shows a representative image of each studied group (I, II and III) of IHC results in CD57 and CD68 cells (Figures 2A,B) as well as IL-10, TNF-α, IL-17 and IFN-γ (Figures 2C-F). The microscopy assessments were carried out separately in the dermis layers including papillary and reticular layers. According to the statistical analysis in each established cytokine (or cell type), no differences of the quantity of immunostained cells were shown between the dermis layers in both groups of patients or in the negative controls. Similarly, no differences were shown between samples from males and females (data not shown).

The distribution of in situ CD68 and CD57 cells as well as cells expressing IL-10, IL-17, TNF-α and IFN-γ was analysed per group of patients (Figure 3). The assays were performed in 23 patients with lesions until 3 months (Group I), 19 patients with lesions after 3 months (Group II) and 14 normal skin samples (Group III). The data are expressed, as mean±standard error of the mean (SEM).

The values of immunostained cells/μm^2 for CD68 were 180.50±9.56 (Group I), 195.6±15.62 (Group II) and 7.7±0.84 (Group III). The values of CD57 cells were 28.55±3.86 (Group I) 14.24±4.65 (Group II) and 1.54±0.35 (Group III).

The values of immunostained cells/μm^2 for IL-10 were 17.78±2.12, 8.52±0.29 and 1.26±0.02 in groups I, II and III, respectively. Likewise, in groups I, II and III, the results for IL-17 were 2.95±0.72, 2.12±0.36 and 0.25±0.06; for TNF-α, 12.29±1.29, 3.08±0.73 and 0.67±0.01; for IFN-γ, 7.61±1.17, 2.81±0.46 and 0.3±0.05.

The statistical analyses showed no difference between groups I and II in expression of CD68 cells and IL-17. On the other hand, the immune expression for CD57 cells, IL-10, TNF-α and IFN-γ in patients from Group I was significantly higher than those patients from Group II.

In the next step, the expression of the IL-10, IL-17, TNF-a, IFN-γ was compared with each group of patients (Figure 4). In Group I, the statistical analyses showed that in situ IL-10 immune expression was significantly higher than IFN-γ (p=.0073) and IL-17 (P<.0001). TNF-α also was significantly higher than IL-17 (p=.0093). In Group II, although the cytokine expression was smaller than Group I, the differences between the cytokines were similar. IL-10 immune expression was significantly higher than IFN-γ and IL-17 (p=.0029) and (P<.0001), respectively. TNF-α also was higher than IL-17 (p=.0037).

4 | DISCUSSION

Based on clinical information, a patient was positive for ACL when the infection was detected by at least two of the four diagnostic assays.[28,30] Supporting this approach, 37.5% of the patients with cutaneous lesions studied here were diagnosed as having ACL. Independent of the time of the lesions, parasites were shown in all 42 biopsies studied in a typical target population for ACL that has been previously well described.[1,7,27,28] The majority were men (67%) of productive age, 15-64 years (80%), with lesions predominantly in arms/hands and legs/feet (67%). The 42 patients had parasites detected in biopsies by positive tests (histopathological/parasitological/PCR, Table 1). After laboratorial results, all patients with ACL were treated with pentavalent antimonial drug.[1,7,27] The cutaneous lesions from the 42 patients were clinically healed by the end of this study. Thus, biopsies were not collected after treatment.

Cellular immunity is critical in ACL, because Th1 and Th2 cytokines are important in hindering development of the infection.[31] Other T-cell

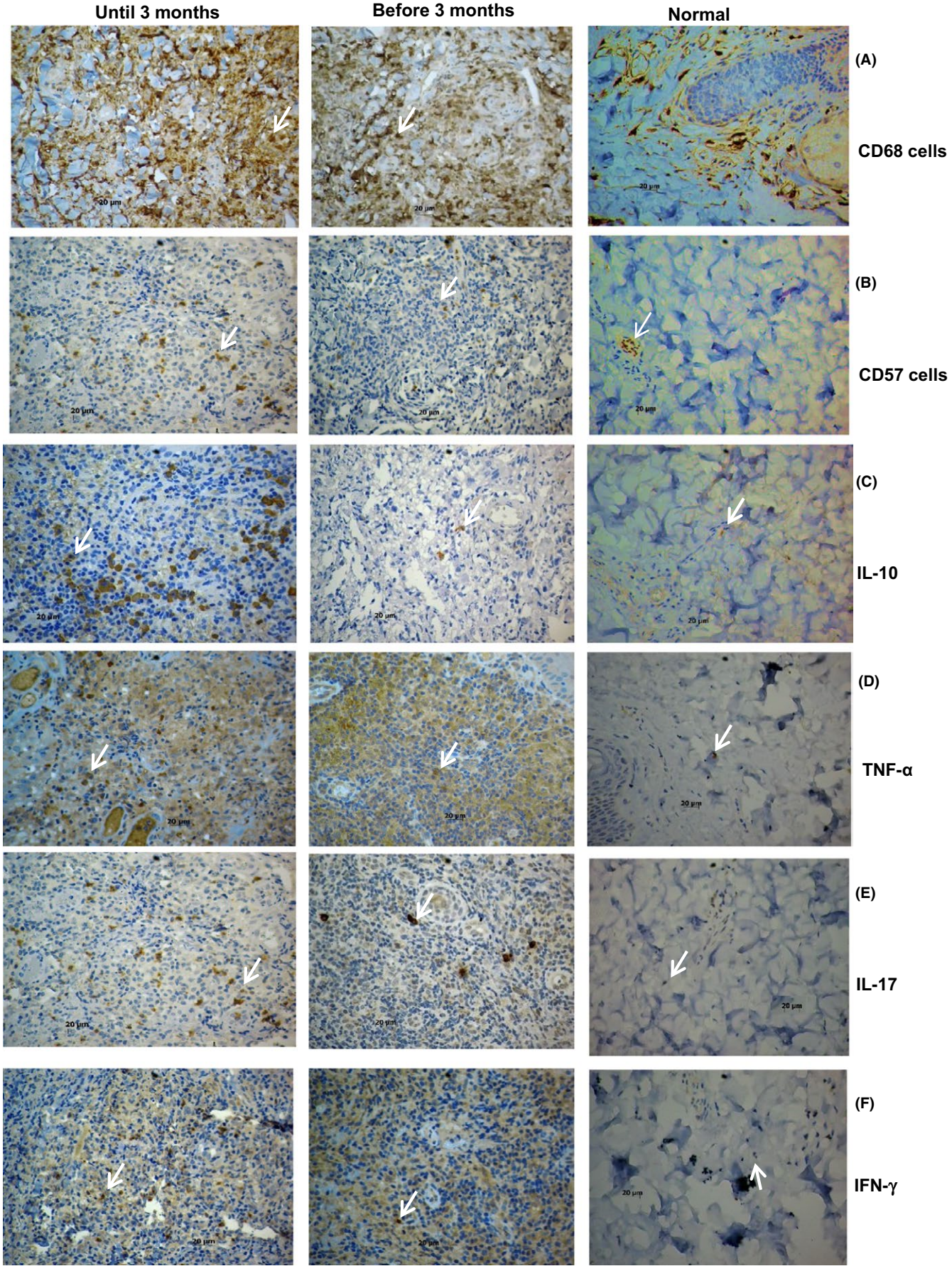
Until 3 months
Before 3 months
Normal
(A)
CD68 cells
(B)
CD57 cells
(C)
IL-10
(D)
TNF-α
(E)
IL-17
(F)
IFN-γ

FIGURE 2 IHC staining in macrophage cells by CD68 expression (A) and lymphoid cells show CD57 expression. (B). Inflammatory cells with expression of IL-10 cells (C), TNF-α (D), IL-17 (E) and IFN-γ (F) in patients from Group I (lesions until 3 months), Group II (lesions after 3 months) and Group III (control). Original magnifications: ×40

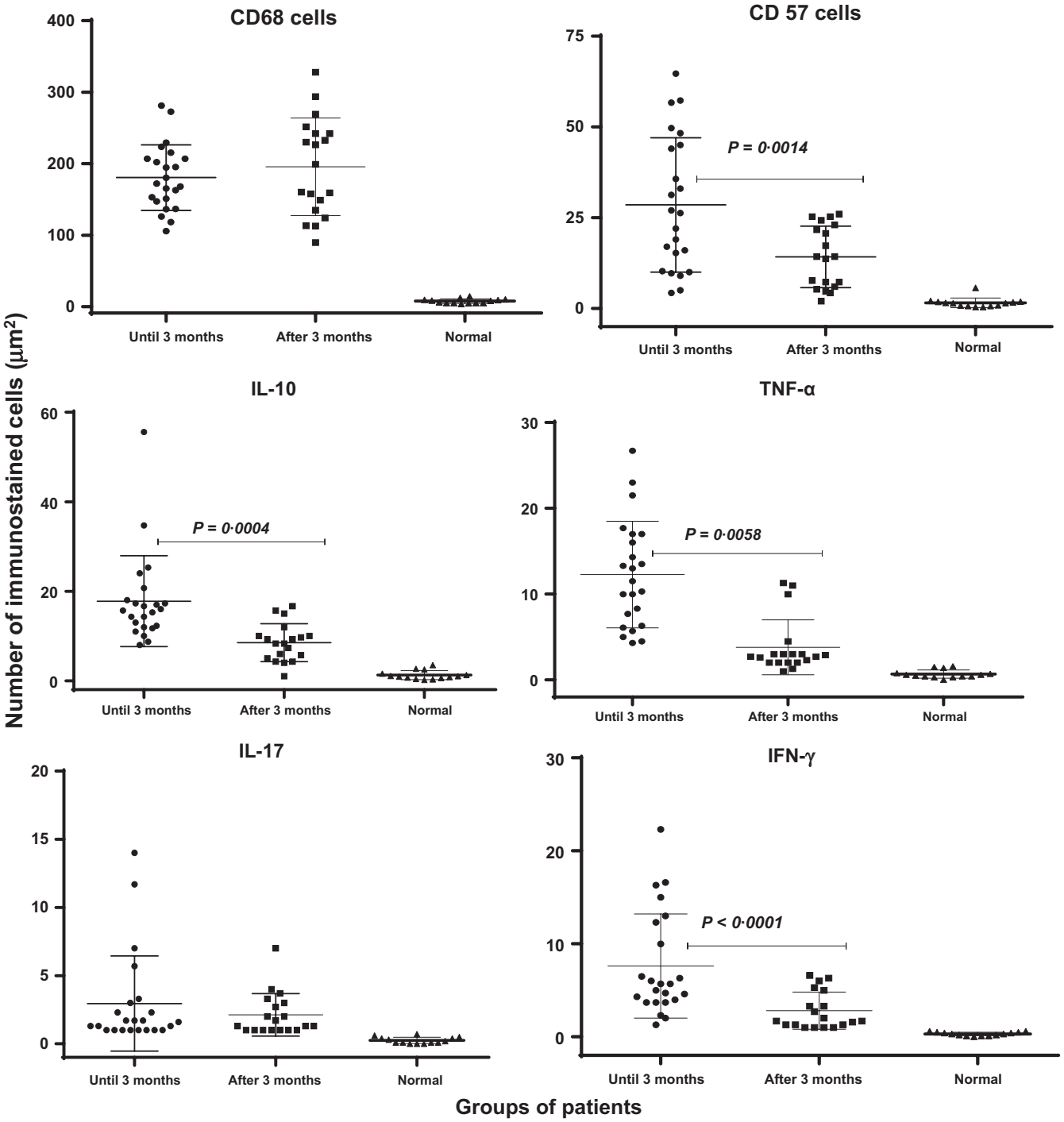

FIGURE 3 *In situ* expression of CD68, CD57, IL-10, TNF-α, IL-17, IFN-γ in biopsies of skin lesions from patients with American cutaneous leishmaniasis (ACL), caused by *Leishmania (Viannia) braziliensis*, with lesions until 3 months, Group I with 23 patients (circles), after 3 months, Group II with 19 patients (squares). The control group (III) with 14 individuals had normal skin (triangles). The methodology was based on IHC as described in the Material and Methods section. The vertical lines indicate the means±SEM. Comparison of the expression between Groups was done by unequal-variance *t* test based on a critical value of $P \leq .05$ and F test to compare variances. Significant differences between Groups are shown in horizontal lines. The results of Group III were statistically different from groups I and II at $P < .0001$

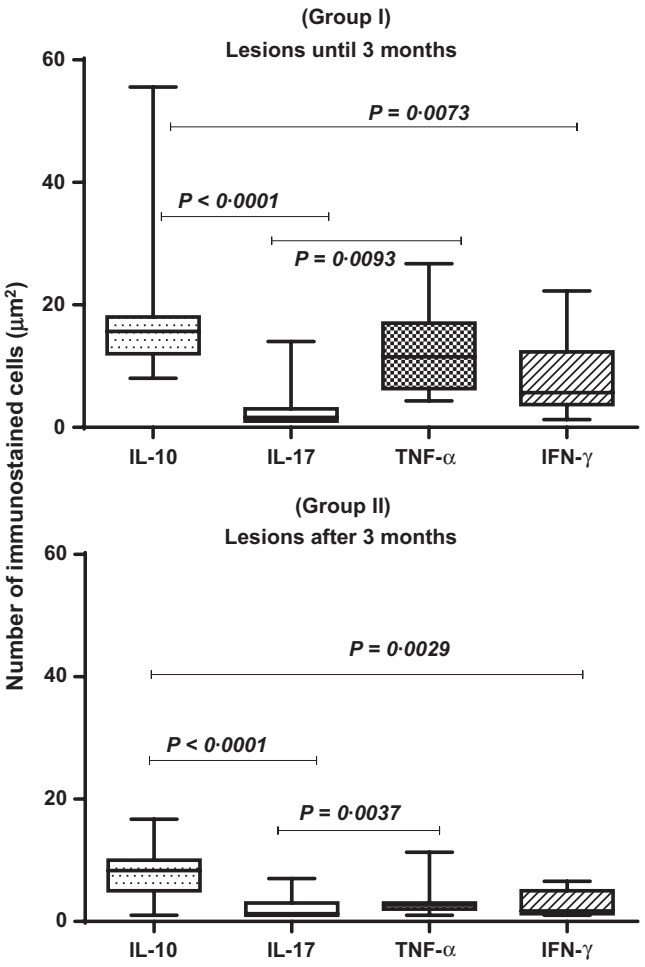

FIGURE 4 Comparison in situ expressions between IL-10, IL-17, TNF-α and IFN-γ in biopsies of skin lesions from patients with American cutaneous leishmaniasis (ACL) are shown above (Group I, 23 patients with lesions until 3 months) and below (Group II, 19 patients with lesions after 3 months). The methodology was based on IHC as described in the Material and Methods section. The vertical lines indicate the means±SEM. Comparison of the expression between cytokines was done by unequal-variance *t* test based on a critical value of $P \leq .05$ and F test to compare variances. Significant differences between cytokines are shown in horizontal lines

subtypes such as Th17 participate in this event, although the exact role of each is not well established.[13,21,32] Such studies were based on analysing blood cells.[10,21,32,33] This study analysed the specific immune response at the site of the infected lesion considered an important tool for defining the evolution of the infection.

For this purpose, the 42 patients with ACL were divided into two groups based on the time of the skin lesions even though amastigotes were detected in biopsies of all of them. One group was in initial phase of ACL (until 3 months), and the other group was composed of patients with late lesions (after 3 months and until 24 months). This group division was based on a previous study of gene expression,[10] in which expression profiles of cytokines and chemokines of patients with recent lesions were different from those with older lesions. The third group used here was composed of skin samples from normal individuals, and the results were used only for the controls in all reactions.

The results of the distribution of in situ CD68, CD57, IL-10, IL-17, TNF-α and IFN-γ showed that except for CD68 and IL-17, patients with recent lesions (Group I) expressed higher levels than those with older lesions. The presence of amastigotes in biopsies causes high levels of antigens and, consequently, cytokine-producing T cells. With respect to TNF-α and IFN-γ, Group I patients expressed more than those from Group II. These data suggest that the presence of these two cytokines and CD57 in the early period of infection can be correlated with the control of parasites as previously described in other studies.[13,22,34–37] IFN-γ synergistically linked to TNF-α activate macrophages to induce the nitric oxide synthase that leads to death of intracellular parasites.[38–40] Consequently, both groups of patients expressed similar concentrations of CD68 cells in order to kill amastigotes in infected cells. However, such cytotoxicity is directly correlated with expressed granzyme causing ulcer development.[20,41,42]

The comparison of cytokine expression per group (Figure 4) showed that IL-10 was significantly higher than IL-17 and IFN-γ. These data suggest an immunological balance between inflammatory-anti-inflammatory agents, and such balance was similar for two groups of patients. High expression of IL-10 at inflamed sites may regulate the host response to tissue damage and lead to later healing.[13,15,21,40] In addition, IL-10 downregulates IFN-γ production.[8,21] The neutralization of IFN-γ decreased the production of TNF-α. In all patients, the balance between TNF-α, IFN-γ and IL-10 appears to be important for controlling the infection and inflammatory process.[8,21] Similar data were shown in other studies, which demonstrate that in more recent lesions, IL-10 appears to be involved in modulation of the immune response.[21]

The immunological balance observed in these patients also was shown in the low expression of IL-17 levels than compared with IL-10 and TNF-α ones. These data also suggested that IL-10 can regulate IL-17.[21] High concentrations of IL-17 cause tissue damage, because they contribute to the pathogenesis of infection. IL-17 properties induce neutrophil recruitment and production of different inflammatory mediators.[21,33,43,44]

Collectively, these data suggested that (i) patients from Group I had recent lesions (in the beginning of chronic phase) compared to those from Group II; (ii) the modulation of inflammatory response in patients with recent ACL was correlated with IL-10 expression in skin lesions[21,33]; (iii) all patients did not develop the strong Th1 immune response with production of high levels of pro-inflammatory cytokines (IFN-γ, TNF-α, IL-17) and low production of IL-10, which promote inflammation with tissue destruction, formation of severe lesions (common in severe cutaneous or mucosal forms)[15,38,39,44,45]; and (iv) all patients, probably, would not develop mucosal forms. In addition, the rapid parasite treatment also prevented the evolution of severe forms. At least, until the end of this study, the 42 patients were considered clinically cured.

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AUTHORS' CONTRIBUTIONS

VL Pereira-Chioccola, AHS Gomes and RB Martines designed the study and experiments, performed the data analysis and interpreted the data. VL Pereira-Chioccola wrote the manuscript. AHS Gomes, CT Kanamura, MLP Barbo and SD Igleziase performed the laboratorial experiments. JA Lauletta Lindoso critically revised the manuscript. AHS Gomes performed the inclusion of patients with American cutaneous leishmaniasis, sample collection and laboratorial diagnosis, interviewed the patients and collected the epidemiological data.

All authors revised the manuscript, approved the final version submitted and published, an agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

CONFLICT OF INTEREST

The authors have no other relevant affiliations or financial involvement with any organization or entire with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript or financial relationships that could be construed as a potential conflict of interest.

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