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In situ study of cellular immune response in human cutaneous lesions caused by *Leishmania (Viannia) panamensis* in Panama

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Abstract

Aims: Leishmaniasis is considered a disease with multiple clinical/immunopathological characteristics, depending on the immunity of the host and the species of the parasite. In Panama, the most prevalent species that causes localized cutaneous leishmaniasis (LCL) is *Leishmania (Viannia) panamensis*, and its immune response is poorly studied. Therefore, we evaluated by immunohistochemistry, the in situ immune response during this infection.

Methods and Results: Biopsies from Panamanian patients with LCL were collected and processed by histological techniques. Infection by *L. (V.) panamensis* was demonstrated by isolation in culture and molecular characterization by Hsp70-RFLP. The in situ immune response was assessed by immunohistochemistry. The immune response was characterized by predominance of T cells, mainly CD8 cells that showed positive correlation with IFN-γ and Granzyme B. CD4 cells presented positive correlation with both IFN-γ and IL-13, pointed by mixed cellular immune response. Regulatory response was characterized by FoxP3 cells, which showed positive correlation to IL-10 but not with TGF-β.

Conclusions: *L. (V.) panamensis* infection triggers a mixed cellular immune response, characterized by the presence of pro-inflammatory, anti-inflammatory and regulatory elements in the skin lesion of Panamanian patients. These data contribute to a better understanding of the immunopathogenesis of *Leishmania Viannia* infection in Panama.

KEYWORDS

cutaneous leishmaniasis, cytokines, immune response, immunohistochemistry, *Leishmania panamensis*, Panama

Kadir Gonzalez the author of this work.

1 | INTRODUCTION

The organisms of the genus *Leishmania* are the causative agents of leishmaniasis, which manifest as a wide spectrum of clinical disease in humans including cutaneous, mucocutaneous and visceral forms.[1] *Leishmania (Viannia)* parasites are the most prevalent aetiologic agents of human cutaneous leishmaniasis (CL) in the Southern and Central America.[2] In Panama, *Leishmania (V.) panamensis* is the most prevalent specie and responsible for cases of localized cutaneous (LCL) and mucocutaneous leishmaniasis (ML).[3] The immune response in leishmaniasis is complex; in some cases, spontaneous cure may occur, and in others, an exacerbated response is observed.[4] The development of a protective immune response requires a coordinated action of the innate and adaptive immunity, which is activated after *Leishmania* sp infection.[4,5] In general, the immune response in leishmaniasis has been mainly characterized by the increase of $CD4^+$ T cells in the early phase of infection, which are able to produce cytokines of Th1 or Th2 profile, depending on specific activation.[6-8] In the host, Th1 response is associated with parasite elimination, by production of pro-inflammatory cytokines like as IFN-γ and TNF-α, while Th2 response is related to parasite survival by anti-inflammatory cytokines like as IL-10, IL-13 and IL-4.[9,10] Human and rodent in vitro studies showed that infection by *L. (V.) panamensis* is characterized by a mixed Th1 and Th2 cellular immune response, mainly with the production of IFN-γ, IL-13, IL-10 and TNF-α; which contributes to disease pathology and frequently follows a chronic course.[1,2,11] In addition to Th1 and Th2 immune response, a regulatory T immune response (Treg) characterized by the transcription factor Foxp3 is also present; which has the main role to regulate the exacerbated immune response maintaining the homeostasis of the immune system.[12] Although Treg cells have been associated to pathology and parasite persistence in leishmaniasis, these cells do not have the same role among infection caused by different *Leishmania* species.[13-15] Studies in *L. (V.) panamensis* infection have demonstrated that Treg cells modulate both the immune response and parasitism.[11] On the other hand, experimental studies showed that depletion of Treg cells during *L. (V.) panamensis* infection leads to disease exacerbation, while temporal enhancement of T reg cells, through cell transfer or antibody therapy, leads to an improvement of disease.[1]

Despite the great importance for public health and the constant scientific efforts in clinical and experimental studies,[2,11,16-21] the understanding of the immune response against *L. (V.) panamensis* infection in Panama is still limited,[2,22] since there are no studies evaluating the in situ immune response in Panamanian patients affected by LCL. In this sense, we characterized the pro-inflammatory, anti-inflammatory and regulatory cellular immune response using immunohistochemistry technique in skin lesions of patients with LCL caused by *Leishmania (V.) panamensis* from Panamá.

2 | MATERIAL AND METHODS

2.1 | Ethical considerations

This study was approved by the National Committee of Bioethics of Research of the Gorgas Memorial Institute of Health Studies, Panama and by the Ethics of Research Committee of the Faculty of Medicine of the University of São Paulo, Brazil, under protocol number 141/13. All the participants signed an informed consent and agreed to participate in the study freely and voluntarily.

2.2 | Study design

Forty-six skin biopsy samples from patients with positive laboratorial and clinical diagnosis of LCL were used. Samples were collected under local anaesthesia and asepsis[23] at the Tropical Medicine Clinic of the Instituto Conmemorativo Gorgas de Estudios de la Salud (ICGES), Panama, between January and December 2012. All patients were adults from endemic areas and without previous treatment for leishmaniasis. Samples were analysed by immunohistochemistry at the Laboratory of Pathology of Infectious Diseases, Medical School, University of São Paulo, Brazil. *Leishmania* infection was confirmed by direct microscopic observation of amastigotes by Giemsa stain and/or isolation of promastigotes in Schneider's medium[23] and immunohistochemistry assay. The *Leishmania* specie was assessed by polymerase chain reaction by kDNA PCR as previously described[24] and characterized as *Leishmania (V.) panamensis* by PCR-Hsp70/RFLP.[25] After diagnosis, all patients were treated with 20 mg/kg/day of intramuscular glucantime according to the Panamanian guidelines for Leishmaniasis control.[26]

2.3 | Sample collection and immunohistochemical procedures

2.3.1 | Biopsy collection

Biopsy specimens were taken with a 4 mm Harris punch (Whatman International Ltd), followed by the application of local anaesthesia and asepsis.[16,27]

2.3.2 | Histopathological processing

All samples were fixed in 10% buffered formalin and processed within a period not more than 48 hours to dispose of the paraffined tissue block. All tissue samples were dehydrated, cleared, embedded in paraffin, cut into 4-5 μm thick sections and prepared to be analysed by immunohistochemistry technique.[27-29]

2.3.3 | Immunohistochemistry

The in situ immune response was assessed by immunohistochemistry using anti-CD4, anti-CD8, anti-CD56, anti-CD68, anti-IL-13, anti-FoxP3 and anti-Granzyme B monoclonal antibodies; and anti-CD3, anti-CD20, anti-iNOS, anti-IL-10, anti-TGF-β1 and anti-IFN-γ polyclonal antibodies. Hyper-immune serum from a mouse chronically infected with *Leishmania (L.) amazonensis*, produced in the Laboratory of Pathology of Infectious Diseases, was used to confirm tissue parasitism.[30,31] Histological sections were deparaffinized in xylene for 15 minutes, followed by hydration with a descending series of alcohols. Endogenous peroxidase was blocked with 3% hydrogen peroxide solution. Antigen retrieval was conducted using 10 mmol/L citrate buffer at pH 6.0 in a boiling water bath. After that, primary antibodies were added to the tissues in the following dilutions: anti-*Leishmania* (mouse hyper-immune serum) diluted at 1:1000, anti-CD3 (A 0452, Dako) diluted at 1:50; anti-CD4 (NCL-LCD4-IF6, Novocastra) diluted at 1:50; anti-CD8 (NCL-L-CD8-295, Novocastra) diluted at 1:50; anti-CD20 [(C-20):SC-7733, Santa Cruz Biotechnology] diluted at 1:1000; anti-CD56 (BCL-L-CD56-504, Novocastra) diluted 1:100; anti-CD68 (ab955, Abcam) diluted at 1:50; anti-iNOS [(N20):SC-651, Santa Cruz Biotechnology] diluted at 1:1000; anti-IFN-γ [(H-145): SC-8308, Santa Cruz Biotechnology] diluted at 1:10; anti-FoxP3 [(H-190): SC-28705, Santa Cruz Biotechnology] diluted at 1:100; anti-IL-13 (08018-3H7, Sigma-Aldrich) diluted at 1:100; anti-TGF-β1 [(V): SC-146, Santa Cruz Biotechnology] diluted at 1:200; anti-Granzyme B (N-terminal, 310215, Sigma-Aldrich) diluted at 1/200; and anti-IL-10 (ab34843, ABCAM) diluted at 1:1000. As a negative control, a solution containing phosphate-buffered saline (PBS) and bovine serum albumin (BSA) with the omission of a primary antibody was used; and human amygdala was employed to standardize the reactions. The slides were incubated in a humidified chamber overnight at 4°C. For all markers, the Novolink kit (RE7280-K-Leica) was used. The chromogenic substrate, DAB + H_2O_2 (diamono-benzidine with hydrogen peroxide—K0690, DakoCytomation), was added to the tissue, incubated for 5 minutes and counterstained with Harris haematoxylin. Finally, the slides were dehydrated in a series of ascending alcohols and mounted with Permount and glass coverslips. Ten skin samples from healthy individuals were also included as controls.

2.3.4 | Quantitative analysis of immunostained cells

Sequential images were obtained using an optical microscope couple to the microcomputer, and quantification of immunostained cells was performed using AxioVision 4.8.2 software (Zeiss, San Diego, CA, USA). The images were obtained in the dermis where the inflammatory infiltrate was observed. Ten microscopic fields of each histological sections for the different markers were photographed using 40X objective. Cells were quantified according to cell morphology and the brown immune-stained, and cellular density (number of cells per square millimetre) was determined by the ratio of the immunolabelled cells to the area of each image.

2.3.5 | Statistical analysis

GraphPad Prism 5.0 software was used for the statistical analysis of the results. To analyse the differences between the groups, the *t* test was performed for the Gaussian distribution data and Mann-Whitney test was used for the non-Gaussian distribution data. In order to correlate the different markers, Pearson's correlation test was performed for data with Gaussian distribution and Spearman's correlation test for data with non-Gaussian distribution. Graphics were made using the Origin 8.0 program.

3 | RESULTS

3.1 | Patients profile

Forty-six samples from positive patients with LCL were analysed; 71,74% (33/46) were male and 28,26% (13/46) female. The mean age was 33 years, ranging between 21 and 72 years. The median of lesions number was 2, ranging between 1 and 8 lesions, with an average of 35 days for the time of evolution, varying from 10 to 90 days. The majority of patients, 65.2% (30/46), had lesions with an evolution time ≤30 days, and 34.8% (16/46) presented lesions >30 days of infection. Lesions were distributed mostly in the upper extremities (63%), followed by lower extremities (18%), face/neck (10%), back (5%) and abdomen (4%). Patients came from recognized leishmaniasis endemic areas in Panama. All parasites isolated from the skin lesions were characterized as *Leishmania (V.) panamensis* by PCR-Hsp70/RFLP.

3.2 | Parasite detection by immunohistochemical analysis

The presence of amastigote forms of *Leishmania* spp. was observed in 78.3% (36/46), and the density (mean ± standard error) of parasites in the samples was 475,6 ± 135,4 amastigotes/mm^2.

3.3 | Histopathological analysis

Were observed morphological alterations both in the epidermis as in the dermis. Alterations of the epidermis were mainly characterized by acanthosis, spongiosis and exocytosis. The presence of ulcers in the epidermis was observed in 43% (20/46) of the biopsies. The histopathological alterations in the dermis were characterized by mononuclear inflammatory infiltrate of variable intensity with diffuse or focal distribution, formed by predominance of lymphocytes, followed by histiocytes and plasma cells. Granulomatous

outline or well-formed epithelioid granulomas presence was observed in 46% (21/46) of the cases, with the presence of multinucleated giant cells. The presence of *Leishmania* amastigote forms in the macrophage cytoplasm was observed in 48% (22/46) of histological sections stained by HE, and the parasitic load varied from mild, moderate to intense.

3.4 | Evaluation of inflammatory and anti-inflammatory immune response

All antibodies used in the present study evidenced positive cells for the markers evaluated in the skin lesion of patients affected by LCL by *L. (V.) panamensis* (Figures S1 and S2). Regarding to the lymphocyte populations observed in the samples infected with *L. (V.) panamensis*, the cell densities were as follows: $CD3^+$ 2593±112 cells/mm^2, $CD4^+$ 914.5 ± 51.76 cells/mm^2, $CD8^+$ 1698±66.84 cells/mm^2, $CD56^+$ 361±38.93 cells/mm^2 and $CD20^+$ 1016±87.94 cells/mm^2 all significantly higher than ones presented in health normal skin (Table S1). Comparative analysis among the cellular markers showed a high $CD3^+$ cell density ($P < .001$), characterized mainly by $CD8^+$ cells in relation to $CD4^+$, $CD56^+$ and $CD20^+$ cells ($P < .05$) (Figure 1A,B). In relation to the macrophage, the mean ± standard error of $CD68^+$ cell density was 963.3 ± 77.94 cells/mm^2 in the skin affected by LCL and 17.80 ± 7.97 cells/mm^2 in the health normal skin (Table S1).

Concerning the intracellular markers, the densities of IFN-γ^+ cells were 840.5 ± 72.73 cells/mm^2, TGF-β^+ cells 132.2 ± 9.50 cells/mm^2, IL-10^+ cells 537.5 ± 30.94 cells/mm^2, $IL13^+$ cells 563.7 ± 42.67 cells/mm^2, $iNOS^+$ cells 808,70 ± 68,20 cells/mm^2 and Granzyme B^+ cells 1019,00 ± 46,91 cells/mm^2 (Table S1), without significant differences among them (Figure 2A). The cellular densities of all intracellular markers used to analyse the inflammatory and anti-inflammatory immune response were significantly higher in the cutaneous lesion of patients affected by LCL when compared to the controls, skin biopsies of healthy skin ($P < .01$) (Figure 2B and Table S1). We did not observe significant difference between the number of cytokines positive cells and tissue parasite load, time of infection, sex or histopathological tissue response (granulomas and ulcer) ($P > .05$), with exception of IFN-γ^+ cells, that presented a higher density in the lesions with the presence of granuloma compared to the lesions without granuloma ($P = .0097$).

To evaluate indicators of disease severity, the ratio between the same markers was calculated for infected and normal skin. The $CD4^+/CD8^+$ cell ratio was 0.54 in the lesions of patients affected by LCL. In contrast, the healthy normal skin showed an inversion of $CD4^+/CD8^+$ cell ratio (2.90). The $CD3^+/CD20^+$ cell ratio did not presented difference between infected and normal skin (2.6 vs 2.87). There was a tendency to higher $CD3^+/CD20^+$ cell ratios in patients with lesions ≤ 30 days of evolution (2.85) when compared to lesions > 30 days of evolution (2.14). In relation to inflammatory and anti-inflammatory cytokines, the IFN-γ/IL-13 ratio was 1.49 and the IFN-γ/IL-10 ratio was 1.56 in the skin lesion of patients affected by LCL; however, in the healthy normal skin they were lower, IFN-γ/IL-13 ratio was 0.97 and IFN-γ/IL-10 ratio was 0.54.

A linear correlation analysis among the markers was also performed. A strong and positive correlation between the number of $CD3^+$ cells and the number of $CD4^+$ and $CD8^+$ cells ($P < .001$), as well as between the number of $CD68^+$ and $iNOS^+$ cells ($P < .001$), was observed. There was a moderate and positive correlation between the number of $CD3^+$, $CD4^+$, $CD8^+$ and $CD56^+$ cells and IFN-γ^+ cells ($P < .001$) (Figure 3). A negative correlation was found, although not significant, between the number of amastigote and the number of $CD8^+$, $CD4^+$ and IFN-γ^+ cells. In addition, a moderate and positive correlation was observed between the number of IL-10^+ cells and the number of $CD8^+$ cells ($P < .001$) and $CD4^+$ cells density ($P < .001$) (Figure 3). Moderate and positive correlation between $CD8^+$ cells and Granzyme B^+ cells ($P < .001$) and between the number of $IL13^+$ cells and $CD4^+$ cells was also observed ($P < .001$). (Figure 3).

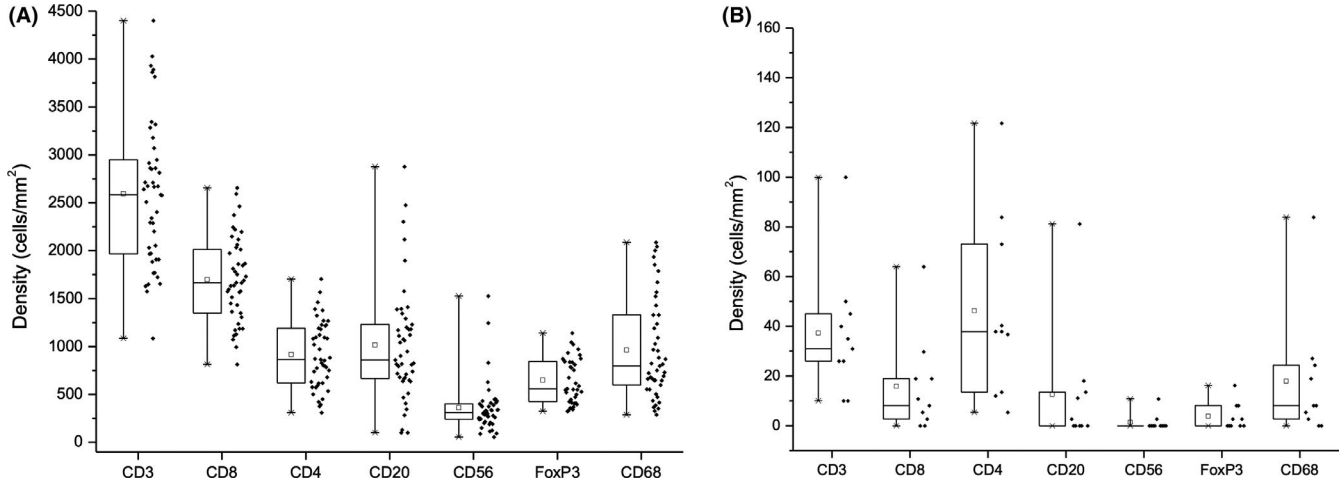

FIGURE 1 Dot plot graphic showing the distribution of positive cells and a box plot showing the median, mean, quartiles, maximum and minimum values for the number of positive cells per square millimetre for CD3, CD8, CD4, CD20, CD56, FoxP3 and CD68 markers in skin lesion biopsies of patients with localized cutaneous leishmaniasis caused by *L. (V.) panamensis* (A) and healthy normal skin (B)

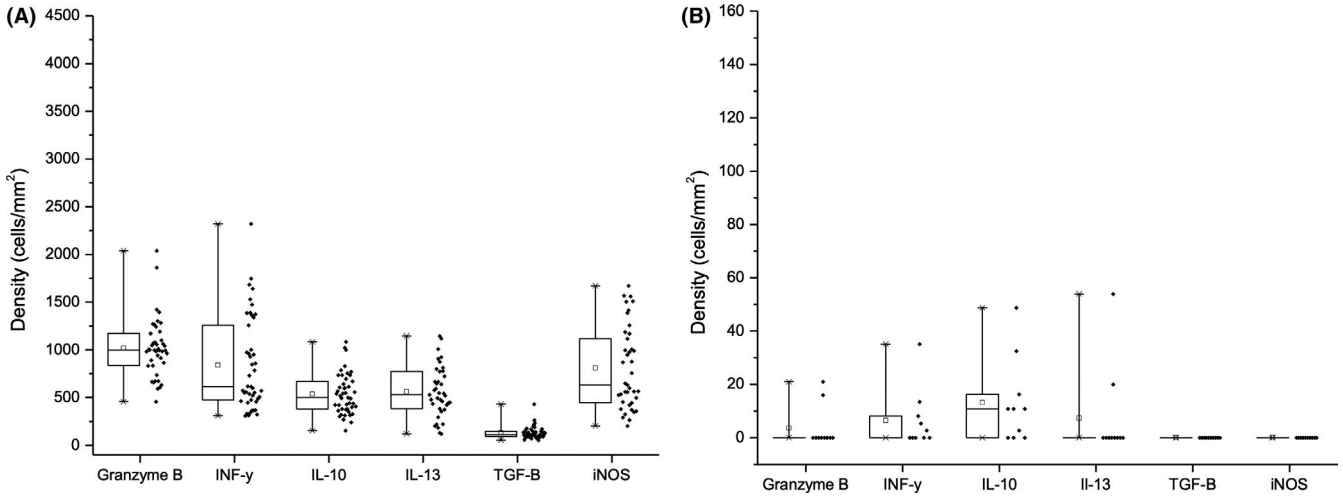

FIGURE 2 Dot plot graphic showing the distribution of positive cells and a box plot showing the median, mean, quartiles, maximum and minimum values for the number of positive cells per square millimetre for iNOS, Granzyme B, IFN-γ, IL-10 and IL-13 markers in skin lesion biopsies of patients with localized cutaneous leishmaniasis caused by *L. (V.) panamensis* (A) and healthy normal skin (B)

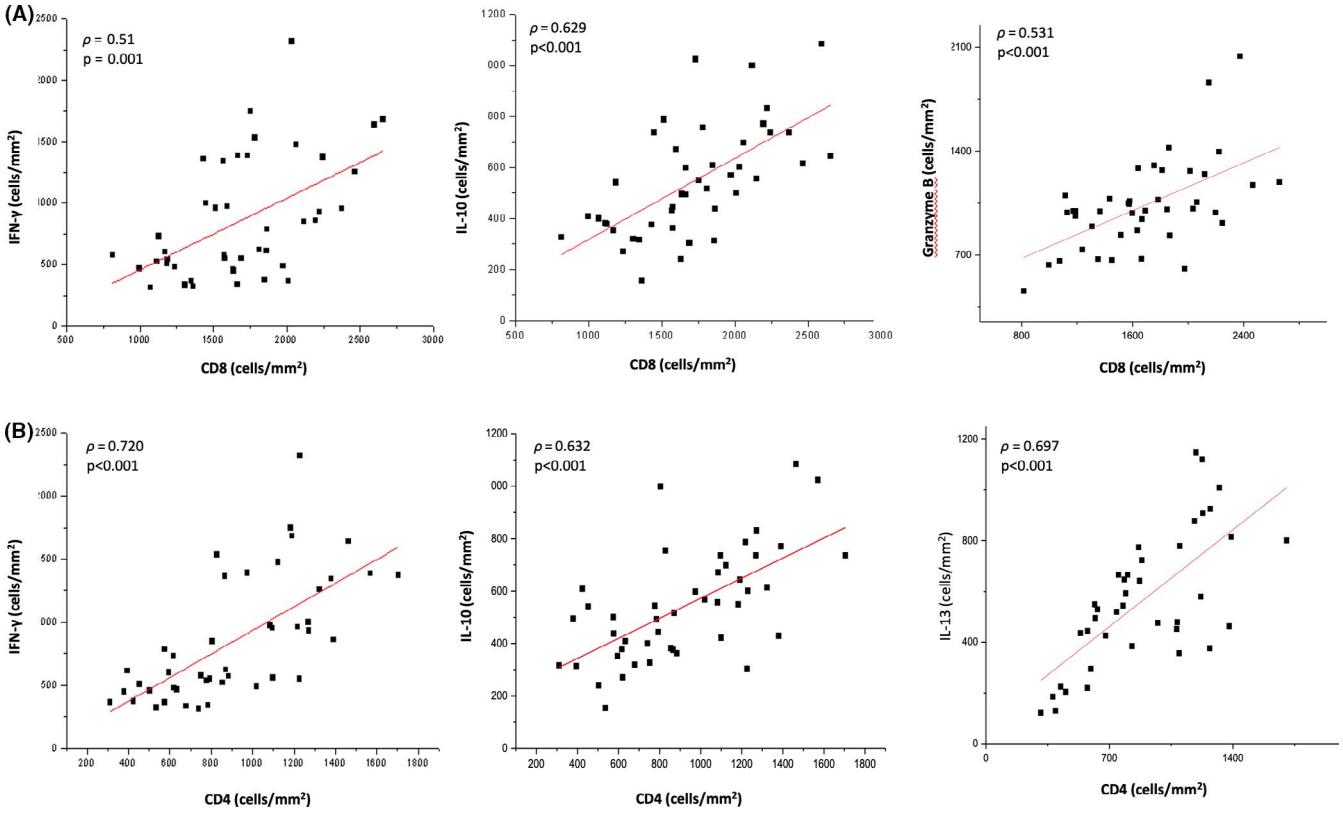

FIGURE 3 Graphic of dispersion showing the correlation between the cellular density of $CD8^+$ cells and IFN-γ^+ cells (A), IL-10^+ cells (B), Granzyme B^+ cells (C), and between $CD4^+$ cells and IFN-γ^+ cells (D), IL-10^+ cells (E), IL-13^+ cells (F). The value of ρ is the Spearman's correlation coefficient, and *P* is the *P* value

3.5 | Evaluation of Regulatory Immune Response

$FoxP3^+$, IL-10^+ and TGF-β^+ were analysed to evaluated the regulatory immune response. Quantitative morphometric analysis showed that the density of $FoxP3^+$ cells was 648.5 ± 38.03 cells/mm^2, IL-10^+ cells 537.5 ± 30.94 cells/mm^2 and TGF-β^+ 132.2 ± 9.50 cells/mm^2 (Figures 1A and 2A). The number of $FoxP3^+$, IL-10^+ and TGF-β^+ cells was higher in the skin biopsies of patients affected by LCL when compared to control healthy skin biopsies ($P < .0001$) (Figures 1B and 2B, Table S1).

The density of the IL-10^+, TGF-β^+ and $FoxP3^+$ cells did not show significant difference in relation to the tissue parasite load, time of

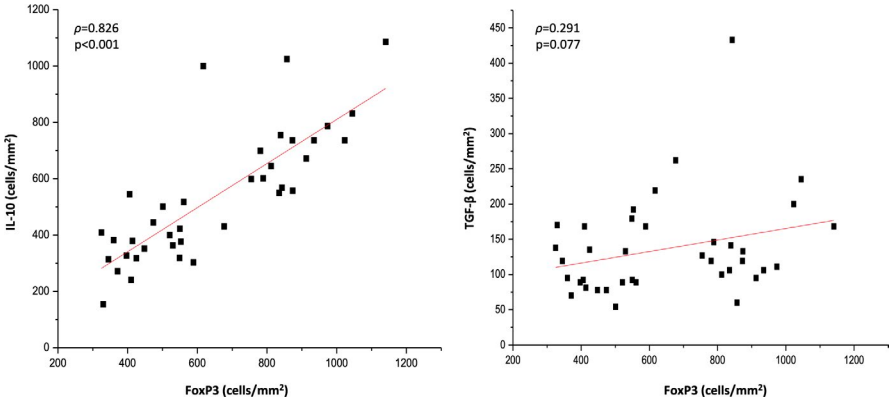

FIGURE 4 Graphic of dispersion showing the correlation between the cellular density of FoxP3$^+$ cells and IL-10$^+$ cells (A), TGF-b$^+$ cells (B). The value of ρ is the Spearman's correlation coefficient, and *P* is the *P* value

evolution of the infection and type of tissue response (presence or absence of granuloma and ulcer) ($P > .05$).

A strong and positive correlation was observed between the density of FoxP3$^+$ and IL-10$^+$ cells ($P < .001$) but it was not observed between the density of FoxP3$^+$ and TGF-β$^+$ cells ($P > .05$). (Figure 4).

4 | DISCUSSION

Concerning to all cellular types evaluated in the present study, lymphocytes (TCD3) were the predominant cells in the dermal lesion site. Predominance of T lymphocytes in the inflammatory infiltrate of the skin lesion biopsies caused by *Leishmania Viannia* infection has been described.[10,17,32,33] Among them, higher density of CD8$^+$ cells was observed. CD8$^+$ cells have been associated with healing and protection in both human and murine leishmaniasis[34-36] through their cytotoxic function as well as IFN-γ production, a potent nitric oxide inducer, mediator that promotes the destruction of parasites.[37,38] In the present study, a positive correlation was observed between CD8$^+$ and IFN-γ$^+$ cells but this correlation was higher between CD4$^+$ and IFN-γ$^+$ cells, suggesting predominant involvement of CD4$^+$ cells in the production of IFN-γ helping the control of tissue parasitism as already has been described for other species of *Leishmania*.[36] On the other hand, a positive correlation was observed between CD8$^+$ and Granzyme B$^+$ cells suggesting their role in tissue damage.[36]

It was observed a participation of Th2 cytokines, like IL-10 and IL-13, that showed direct correlation to CD4$^+$ cells. These cytokines are involved in maintaining the anti-inflammatory response and consequently the tissue parasite persistence.[2,39] In addition, a positive correlation of CD8$^+$ and IL-10$^+$ cells suggests that a proportion of CD8$^+$ cells may be producing IL-10 contributing to the anti-inflammatory response, as has been showed in in vitro studies with *L. (V.) guyanensis*.[40]

As an indicator of disease severity, the CD4/CD8 cell ratio was evaluated. A decrease in CD4/CD8 ratio was observed (0.56) when compared to healthy skin (2.90), evidencing the increase of CD8$^+$ cells in the skin biopsy caused by *L. (V.) panamensis*. Similar results were observed in LCL caused by *L. (V.) braziliensis* that present a CD4/CD8 ratio < 1, and it was related to disease severity.[41-43] However, an increase in CD4/CD8 ratio was observed in the skin lesion of patients with LCL caused by *L. (V.) braziliensis* after specific treatment.[38] Another interesting study conducted in Bolivia, patients infected by *L. Viannia* demonstrated that the CD4/CD8 ratio was higher in patients that failed to treatment response than in patients with a good response to treatment.[42] These data are useful to understand the immunopathology of CL caused by different species of *Leishmania* and should be considered in future therapeutic and management studies.

Regardless of the cell type involved in the inflammatory infiltrate, it is important to evaluate the type of cellular immune response that these cells are involved. In this sense, we evaluate the inflammatory or anti-inflammatory immune response in patients with LCL caused by *L. (V.) panamensis* through the number of positive cells for cytokines as well as the ratio of IFN-γ/IL-10 and IFN-γ/IL-13. We observed that the ratio of IFN-γ/IL-10 was 1.56, and the ratio of IFN-γ/IL-13 was 1.49, suggesting a predominance of inflammatory immune response. It is important to mention that NK cells are another important source of IFN-γ [38,44]; but in the present study low number of CD56$^+$ cells were observed. However, a direct correlation between CD56$^+$ and IFN-γ$^+$ cells was observed; therefore, in spite of slight involvement of these cells in the skin lesion caused by *L panamensis*, its role cannot be discarded.[45,46]

Macrophage activation through inflammatory cytokines, specially INF-γ, is associated with disease control by production of nitric oxide (NO) and reactive oxygen species (ROS), which are the main mediators responsible for parasite death.[45,47] Our data showed a moderate presence of CD68$^+$ cells, but a strong and direct correlation with the number of iNOS$^+$ cells, suggesting the presence of activated macrophages. However, the presence of inactivated macrophages at the site of injury may be also associated with parasite persistence and maintenance of inflammation.[45,48] Despite the presence of cells with morphological characteristics of macrophages and iNOS$^+$, macrophages without iNOS expression were also observed, which can be considered important host cells for the parasite multiplication and survival. In this sense, positive correlation between the number of *Leishmania* amastigote and CD68$^+$ cell was observed, similar to the study reported by Saldanha et al[49] in LCL by *Leishmania Viannia* parasites.

The number of amastigotes is high in the initial papular lesions, and it decrease during the process of ulceration, indicating that the ulcers formation is associated with a reduction in the parasitic load at the site of skin lesion.[49] However, in our study no significant difference was observed between the number of amastigotes and the time of evolution of the lesion, and the ulcers formation. According to Saldanha et al, the lack of association between the parasitic load, the area of necrosis and the size of the ulcer suggests that the parasitic load does not play a direct role in the development of the lesion. Therefore, it is likely the proposed statement that the parasite's antigens derived from dead amastigotes are able to stimulate the adaptive immune response, thus increasing the inflammatory reaction.[49]

Histological studies revealed a large amount of B cells in the inflammatory infiltrate of skin lesions of Colombian patients affected by LCL caused by *L. (Viannia)*.[17] In our study, a considerable amount of B cells ($CD20^+$ cells) was observed, and the $CD3^+/CD20^+$ cell ratio tended to decrease with the time of evolution of the lesion. However, other authors have described an increase in the number of B cells during the chronic phase of infection caused by *L. (Viannia)* in murine and human cutaneous leishmaniasis.[2,50] Another study reported that B-cell infiltration at the site of infection was correlated with the development of pathology in human *L. (V.) panamensis* infection.[17,51] Therefore, it is suggested that B cells contribute to the pathology of *L. (V.) panamensis* disease, but further studies are needed to evaluate in detail the role of these cells type in LCL caused by *L. (V.) panamensis*.[2]

Species belonging to the subgenus *Viannia* are known to induce intense inflammatory response in patients with LCL and, therefore, immunomodulatory mechanisms are necessary for the control of the inflammatory process.[1] The presence of Treg cells in the skin lesion may be related to the control of the exacerbated inflammatory response and pathology of the disease. The activation of Treg cells occurs through a direct interaction between antigen-presenting cells and T cells, which can be modulated by the presence of cytokines such as IL-10 and TGF-β.[12,52] Although it is known that Treg cells may contribute to the persistence of the parasite in leishmaniasis, they do not play an equal role in infection by different *Leishmania* species.[53] Recently, it has been described that patients infected with *L. (V.) panamensis* showed decreased Treg cell functionality during infection, but their suppressive capacity was restored after successful treatment.[11] It has also been reported that experimental depletion of Treg cells during infection led to disease exacerbation and also that temporal increase of Treg cells leads to the control of the disease by pointing out the beneficial role of these cells.[1] In this way, a minor pathology associated with the regulatory activity of Treg cells through IL-10 was described in viral infection. The FoxP3 cells apparently may limit fibrogenesis in hepatitis C infection by IL-10 produced by Treg in comparison with TGF-β produced by Treg cells.[54] In this study, we demonstrated the presence of Treg cells in patients with LCL by FoxP3-positive marker, but we did not evidence their protective role.

Natural and adaptative or inducible Treg cells have been described.[55,56] The inducible Treg cells were subdivided into 3 groups: T regulatory 1 (Tr1) which secrete IL-10, T helper 3 (Th3) which secrete TGF-β and converted Treg FoxP3.[55,56] Despite IL-10 can be produced by natural Treg, inducible Tr1 cells are the relevant source of this cytokine during infection.[56,57] Although TGF-β seems to play an important role in the maintenance and development of Treg,[58] we observed a positive correlation between $FoxP3^+$ cells and $IL\text{-}10^+$ cells, but no correlation was showed with $TGF\text{-}\beta^+$ cells. These data suggest that Treg cells are regulating mainly by the action of IL-10 as shown in studies with *L. (V.) guyanensis* where the suppressive role of Treg cells is mainly mediated by the action of IL-10.[40,59] Probably, we have inducible Tr1 cells present in *Leishmania* infection like others infections caused by bacteria, parasite and virus.[56,57,60,61] IL-10 producing Tr1 cells have also been identified in visceral leishmaniasis and related to prevent immune pathology and tissue damage, but they have also related to promote the establishment of infection.[62,63]

Taking together, our data showed that LCL caused by *L. (V.) panamensis* triggers a mixed of cellular immune response, characterized by the presence of pro-inflammatory, anti-inflammatory and regulatory elements in the skin lesion. Despite the presence-of $IFN\text{-}\gamma^+$ cells and $iNOS^+$ cells that could be contributing to the control of tissue parasitism, there is evidence of tissue damage and persistence of the parasite, probably related to cytotoxic cells and anti-inflammatory cytokines. This mixed cellular immune response has been related to clinical pathology observed in *L. (V.) panamensis* infection.[1] The findings obtained will contribute to a better understanding of the pathogenesis of this infection and the development of new strategies for the control and treatment of CL in this country.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS

KG, ML, AS, JEC and CC contributed to the acquisition of the data and designed the study. KG, TT, CG, CS and GA performed the experiments. KG and ML drafted the manuscript. KG, AS, JEC and ML critically helped to interpret the data and revised the manuscript. All authors agreed to be fully accountable for ensuring the integrity and accuracy of the work and read and approved the manuscript.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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