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Characterization of regulatory T cell (Treg) function in patients infected with *Leishmania braziliensis*

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Abstract

Th1 immune responses are crucial for eliminating *Leishmania* parasites. However, despite strong Th1 responses, cutaneous leishmaniasis (CL) patients infected with *L. braziliensis* develop the disease, while milder Th1 responses are found in sub-clinical (SC) infections. Therefore, CL patients may experience impaired regulatory T cell (Treg) function, causing excessive Th1 responses and tissue damage. To address this hypothesis, we characterized the function of circulating Tregs in *L. braziliensis* infected CL patients and compared them to Tregs from uninfected controls (UC) and SC subjects. The frequency of circulating Tregs was similar in CL patients, UC and SC subjects. Moreover, CL patients Tregs suppressed lymphocyte proliferation and PBMC pro-inflammatory cytokine production more efficiently than UC Tregs, and also produced higher levels of IL-10 than UC and SC Tregs. Furthermore, PBMC and mononuclear cells from lesions of CL patients responded normally to Treg-induced suppression. Therefore, the lesion development in CL patients infected with *L. braziliensis* is not associated with impairment in Treg function or failure of cells to respond to immunomodulation. Rather, the increased Treg activation in CL patients may impair parasite elimination, resulting in establishment of chronic infection. Thus, immunological strategies that interfere with this response may improve leishmaniasis treatment.

Keywords

Regulatory T cells; *Leishmania braziliensis*; cutaneous leishmaniasis

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Conflicts of Interest

The authors declare no financial or commercial conflicts of interest.

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1. Introduction

In humans and experimental mouse models, the development of a Th1 response is crucial for eliminating or controlling *Leishmania* parasites [1–3]. Patients with active cutaneous leishmaniasis (CL) due to *Leishmania braziliensis* infection develop a strong cellular immune response to parasite antigens and produce high levels of IFN- γ and TNF- α . However, despite the presence of this classic protective immune response, the patients do not control the infection, which culminates in lesion development [4–6]. Curiously, approximately 70% of people infected with *L. braziliensis* develop sub-clinical (SC) disease, and they display milder IFN- γ and TNF- α production in response to *Leishmania* antigen compared to CL patients [7]. Moreover, the drugs that down modulate the immune response, such as pentoxifylline, accelerate the healing of CL and mucosal leishmaniasis (ML) lesions [8]. Therefore, it is possible that the magnitude of the immune response in SC subjects is sufficient to control the infection without causing lesions, while the inflammatory response in CL patients is not appropriately down modulated, resulting in excessive Th1 responses and tissue damage. Such a failure to down modulate pro-inflammatory responses suggests impaired efficacy of regulatory T cells (Tregs). Tregs are able to suppress cell activation and pro-inflammatory cytokine production by a variety of leukocyte populations [9], and were shown to have an important role in the modulation of specific Th1 responses in experimental leishmaniasis [10–12]. Moreover, these cells are also found in the lesions of patients with CL due to *L. braziliensis* infection [12], and therefore we asked if Tregs were able to differentially suppress the Th1 immune responses in CL patients, SC subjects and UC. Our results showed that, contrary to our original assumptions, the Tregs of CL patients suppressed T cell proliferation and PBMC pro-inflammatory cytokine production more effectively than the uninfected control (UC) Tregs and that PBMC from CL patients responded normally to Treg suppression. Moreover, CL patients Tregs produced higher levels of IL-10 compared to the UC or SC subject Tregs, suggesting that the milder Th1 immune response observed in SC patients is not due to Treg-mediated down modulation. Thus, our results suggest that *L. braziliensis* may exploit the host immune response by activating Treg suppressor activity and IL-10 production, which favors parasite persistence. Consequently, *L. braziliensis* promotes the progression to chronic disease and local inflammation.

2. Material and methods

2.1. Cutaneous leishmaniasis patients and uninfected controls

PBMC and skin biopsies were obtained from CL patients attending either at the health post from Corte de Pedra village, Bahia, Brazil, or at the Ribeirão Preto Medical School Hospital, University of São Paulo. The criteria for inclusion in the study were the presence of leishmaniotic ulcers, not localized to mucosal regions, positive Montenegro skin test (≥ 5 mm) and parasite isolation or parasite DNA detection from lesion smears. A total of 45 CL patients were used (33 men and 12 women, 8 to 67 years old) (Table 1). PBMC from 28 uninfected donors were used as controls (13 men and 15 women, 22 to 53 years old). 6 SC subjects samples were obtained from household contacts of CL patients living in the endemic area from Corte de Pedra who had a positive Montenegro skin test or evidence of IFN- γ production in lymphocyte cultures stimulated with *Leishmania* antigens, 3 to 5 years prior to this study and remained without disease (2 men and 4 women, 19 to 75 years old) (Table 1). All procedures with humans were approved by the ethics committees from the University Hospital Complex Edgar Santos, Federal University of Bahia and the Ribeirão Preto Medical School, University of São Paulo.

2.2. PBMC and lesion mononuclear cell isolation

PBMC from CL patients, SC subjects and UC were isolated from buffy coats obtained by centrifuging whole blood through a Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden) gradient. The CL biopsies were collected from the border of the lesions using a 4-mm biopsy punch, and digested in serum-free RPMI medium (Sigma Aldrich, Saint Louis, Missouri, USA) with 500 µg/mL liberase CI (Roche, Basel, Switzerland) for 1 hour at 37°C. They were then macerated in medcons (BD Biosciences, San Diego, California, USA). The cell suspension was centrifuged through a Ficoll-Hypaque gradient (GE Healthcare), and the mononuclear cells were isolated and quantified.

2.3. CD4⁺ T lymphocyte immunophenotyping

PBMC from CL patients, SC subjects and UC were stained for CD4, CD25, CD210 (IL-10R), CD127 and CCR4 and FoxP3, using specific antibodies labeled with FITC, PE, PE-Cy7, APC and Alexa Fluor 750 (all from BD and eBiosciences - San Diego, California, USA). The Human FoxP3 Buffer Set was used for intracellular FoxP3 staining (BD). The samples were acquired in a FACSCanto II flow cytometer (BD), and the analyses were performed using the FACSDiva (BD) and Flow Jo softwares (TreeStar, Ashland, OR, USA).

2.4. Isolation of CD4⁺CD25⁺ cells and co-cultures

CD4⁺CD25⁺ cells were purified from the total PBMC using the CD4⁺CD25⁺ Regulatory T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. For the co-cultures, the total PBMC or lesion infiltrating mononuclear cells were stained with CFSE and cultured in the presence of purified CD4⁺CD25⁺ cells at 1:10 or 1:5 PBMC. A total of 2×10^5 cells were added per well in flat-bottomed 96-well plates and cultured with or without 2.5 µg/ml anti-CD3 (BD) or SLA (soluble *Leishmania* antigen) (5 µg/ml), in complete RPMI medium (supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.1 mM non-essential amino acids and 50 µM β-mercaptoethanol) (Gibco, Grand Island, New York, USA).

2.5. T cell proliferation measurement

Cells were cultured for 96 hours at 37° C in a 5% CO₂ atmosphere, and CFSE⁺ cells were analyzed regarding staining dilution. For each sample, CFSE dilution was evaluated in anti-CD3-stimulated cultures, in the presence or absence of different amounts of CD4⁺CD25⁺ purified cells. For each sample and co-culture condition, controls were performed in the absence of stimulus, and cell proliferation was absent or negligible. The samples were acquired in a FACSCanto II flow cytometer (BD) and analyzed in FACSDiva (BD) and Flow Jo softwares (TreeStar).

2.6. Cytokine production measurements

For measuring cytokine production by CL patients', SC subjects' and UC PBMC, or by CL lesion infiltrating mononuclear cells, in co-culture experiments, half of the supernatant of cultures used in proliferation experiments was removed after 72 hours of culture, and IFN-γ, TNF-α and IL-17 concentrations were measured using the Cytometric Bead Array (CBA) technique (BD). Cytokine production was measured in anti-CD3 and SLA stimulated cells. For each sample and co-culture condition, controls were performed in the absence of stimulus, and cytokine production was below detection threshold. The samples were acquired in a FACSCanto II cytometer (BD) and analyzed using FCAP Array software (Soft Flow, Burnsville, Minnesota, USA). To detect intracellular cytokines, 5×10^5 cells/well were cultured in 96-well flat-bottomed plates at 37°C and 5% CO₂ for 72 hours in complete RPMI medium in the presence of SLA (5 µg/ml). Next, the supernatant was removed, and

the cells were stimulated with PMA (10 ng/ml) and ionomycin (500 ng/ml) for additional 6 hours, in the presence of brefeldin. The cells were stained for surface molecules and then permeabilized using the Cytotfix/Cytoperm kit (BD), following the manufacturer's instructions. IL-10 and TGF- β were stained with specific antibodies conjugated to PE and APC (BD and R&D systems, Minneapolis, Minnesota, USA). The samples were acquired in a FACSCanto II flow cytometer (BD) and analyzed in FACSDiva (BD) and Flow Jo softwares (TreeStar).

2.7. Statistical analyses

The statistical analyses were performed in GraphPad prism 5.0 software (GraphPad Software, San Diego, California, USA). The tests used were the two-tailed non-parametric Mann-Whitney test and the two-tailed Wilcoxon matched-pairs signed rank test. P values < 0.05 were considered significant for all analyses.

3. Results

3.1. Frequencies of Tregs in the PBMC of CL patients

We first examined the Treg frequency in the PBMC of CL patients compared to UC and SC subjects. The frequencies of CD4⁺CD25⁺, CD4⁺CD25^{high} and CD4⁺CD25^{low} T cells were similar among the studied groups (Fig. 1A). The frequencies of CD4⁺CD25^{high} T cells that expressed FoxP3⁺ and CCR4⁺ were similar in all groups studied, but CD127⁺ cells were lower in SC subjects compared to UC and CL patients (Fig. 1B). To determine if the excessive pro-inflammatory response found in CL patients was associated with impaired responses to IL-10, we assayed the expression of the IL-10 receptor (IL-10R) in CD4⁺ T lymphocytes. We found that the IL-10R expression was similar in all of the groups studied (Fig. 1A), suggesting that the CD4⁺ T cells from CL patients must present unimpaired response to IL-10.

3.2. Circulating CD4⁺CD25⁺ T cells from CL patients efficiently suppress T cell proliferation

To evaluate the suppressive capacity of CD4⁺CD25⁺ T cells from CL patients on T cell proliferation, we cultured the CD4⁺CD25⁺ T cells with PBMC from UC or CL patients in the presence of anti-human CD3. The same procedure was performed using CD4⁺CD25⁺ T cells from UC, and the suppressive capacity of CD4⁺CD25⁺ T cells from both groups were compared. Culturing the PBMC with CD4⁺CD25⁺ T cells from the CL patients resulted in decreased CL and UC T cell proliferation (Figs. 2A and 2B). The suppression of T lymphocyte proliferation was also observed when UC CD4⁺CD25⁺ cells were cultured with total CL patients' and UC PBMC (Figs. 2C and 2D). The CD4⁺CD25⁺ cells from CL patients more efficiently suppressed the proliferation of CL patients' PBMC T lymphocytes at a 1:10 ratio compared to the UC CD4⁺CD25⁺ cells ($p = 0.03$). In all of the other conditions tested, the suppressive capacities of the CL patients' and UC CD4⁺CD25⁺ cells were similar (Table 2 – vertical p values).

We also assessed whether the T lymphocytes from CL patients' or UC PBMC were differentially susceptible to the CD4⁺CD25⁺ T cell-mediated suppression of the proliferative response. The proliferation of T lymphocytes from CL and UC PBMC was suppressed with similar intensity in the presence of CD4⁺CD25⁺ T cells from CL patients, and similar data were observed in the presence of UC CD4⁺CD25⁺ T cells (Table 2 – horizontal p values). These results show that the CD4⁺CD25⁺ T cells from CL patients suppressed T lymphocyte proliferation more efficiently than the UC CD4⁺CD25⁺ T cells, and the T lymphocytes present in CL patients' PBMC responded normally to the suppression of lymphocyte proliferation exerted by Tregs.

3.3. Circulating CD4⁺CD25⁺ T cells from CL patients suppress the production of pro-inflammatory cytokines

We also evaluated the ability of CD4⁺CD25⁺ T cells from CL patients to suppress the production of pro-inflammatory cytokines. For this, we quantified the production of IFN- γ , TNF- α and IL-17 in the supernatants of CD4⁺CD25⁺ T cells and total PBMC co-cultures stimulated with -CD3. We observed that circulating CD4⁺CD25⁺ T cells from CL patients were able to suppress the production of IFN- γ , TNF- α and IL-17 by the PBMC from CL patients and UC in all of the co-culture conditions tested (Figs. 3A, B and C). In the presence of purified UC CD4⁺CD25⁺ cells, the production of these cytokines by CL patients' and UC PBMC were significantly suppressed in all of the conditions tested (Figs. 3A and 1B), with the exception of IL-17 production by UC PBMC (Fig. 3C).

At a 1:10 co-culture ratio, the CL patients' and UC CD4⁺CD25⁺ cells similarly suppressed the production of IFN- γ , TNF- α and IL-17 from the CL patients' and UC PBMC. Additionally, the CL patients' and UC CD4⁺CD25⁺ cells similarly suppressed the production of these cytokines by the UC PBMC at a 1:5 co-culture ratio (Figs. 3D, E and F). However, the production of TNF- α (Fig. 3E) and IL-17 (Fig. 3F) by the CL patients' PBMC were more efficiently suppressed by the CL patients' CD4⁺CD25⁺ T cells at 1:5 co-culture ratio, while no difference in the suppression of IFN- γ production was observed in the presence of CL patients' or UC CD4⁺CD25⁺ T cells (Fig. 3A).

We next evaluated whether the CL patients' or UC PBMC were differentially susceptible to Treg-mediated cytokine production suppression by comparing the level of suppression after culturing CL patients' and UC PBMC with CD4⁺CD25⁺ cells. The productions of IFN- γ (Fig. 3G), TNF- α (Fig. 3H) and IL-17 (Fig. 3I) were similarly suppressed in PBMC from CL patients and UC when they were cultured with CL patients' CD4⁺CD25⁺ T cells. Additionally, the production of these cytokines was equally suppressed in the CL patients' and UC PBMC when they were cultured with UC CD4⁺CD25⁺ T cells.

However, to successfully limit inflammation during the infection, the CD4⁺CD25⁺ T cells must exert control on antigen specific T cells responses. We therefore compared the ability of CL patients' and UC CD4⁺CD25⁺ T cells to suppress the production of pro-inflammatory cytokines by CL patients PBMC that were stimulated with *L. braziliensis* antigen. The CL patients' and UC CD4⁺CD25⁺ T cells were able to suppress the production of IFN- γ (Supplemental Fig. 1A) and TNF- α (Supplemental Fig. 1B) in both the co-culture ratios tested, while IL-17 production was suppressed by CL patients' CD4⁺CD25⁺ T cells at both ratios tested and by UC CD4⁺CD25⁺ cells only at the 1:10 co-culture ratio (Supplemental Fig. 1C). However, the CL patients' CD4⁺CD25⁺ T cells more efficiently suppressed the production of IFN- γ (Fig. 4A) and IL-17 (Fig. 4C) than the UC CD4⁺CD25⁺ cells, while the suppression of TNF- α production was similar between CL patients' and UC CD4⁺CD25⁺ cells (Fig. 4B). The same procedures were performed using UC PBMC stimulated with SLA, however, the levels of cytokines produced were comparable to non-stimulated control cultures (results not shown).

Taken together, these results show that the CL patients' CD4⁺CD25⁺ T cells were more efficient at suppressing the production of pro-inflammatory cytokines compared to the UC CD4⁺CD25⁺ T cells, and the effector cells from CL patients were responsive to the suppression of pro-inflammatory cytokines exerted by the Tregs.

3.4. Comparison of CD4⁺CD25⁺ T cell function between CL and SC subjects

Because SC subjects are infected but do not develop lesions, it is possible that their immune response is effective and properly modulated, resulting in the absence of tissue damage. We

therefore evaluated whether the CD4⁺CD25⁺ T cells from SC subjects were more effective at suppressing T cell proliferation and PBMC cytokine production than the CL patients' CD4⁺CD25⁺ T cells.

We observed that the CL patients' and SC subjects' T lymphocyte proliferation was similarly inhibited by CL patients' or SC subjects' CD4⁺CD25⁺ T cells (Fig. 5A). We also showed that IFN- γ production by CL patients' and SC subjects' PBMC was similarly decreased upon co-culture with CL patients' and SC subjects' CD4⁺CD25⁺ T cells (Fig. 5B), and the same results were observed for TNF- α production (Fig. 5C). This result suggests that the development of lesions in patients infected with *L. braziliensis* was not related to the functional status of their Tregs.

3.5. CL patients CD4⁺CD25⁺ T cells actively suppress lesion infiltrating mononuclear cells

We next asked if Tregs were able to modulate the lesion infiltrating mononuclear cells. To evaluate this, mononuclear cells isolated from the lesions of CL patients were stimulated with SLA in the presence or absence of CD4⁺CD25⁺ cells from CL patients' PBMC, and the production of IFN- γ and TNF- α quantified. We found that IFN- γ and TNF- α were produced by lesion total mononuclear cells, and both cytokines were inhibited by CL patients' CD4⁺CD25⁺ cells. The amount of IL-17 produced in stimulated cells was not different from that obtained from the supernatants of non-stimulated cells. The production of IFN- γ and TNF- α was also inhibited in the presence of CL patients' CD4⁺CD25⁺ cells when anti-CD3 was used as a polyclonal stimulus for the mononuclear lesion cells (not shown). These results once again demonstrated that the Tregs from CL patients were capable of effectively modulating the peripheral immune responses.

3.6. CD4⁺CD25⁺ T cells from CL patients produce higher levels of IL-10

To investigate how the CD4⁺CD25⁺ T cells exert their suppressive activity, we performed intracellular staining in CD4⁺ T cells, of IL-10 and TGF- β , whose production are important modulatory mechanisms used by Tregs. The percentage of IL-10⁺ CD4⁺ cells was higher in CL patients' compared to UC and SC subjects' PBMC, and there was no difference between UC and SC subjects' cells (Fig. 7A). The same result was observed in CD4⁺CD25⁺ T cell (Fig. 7B) and CD4⁺CD25⁻ T cell subpopulations (Fig. 7C), where CL patients presented higher proportion of IL-10⁺ cells compared to UC and SC subjects, and no difference was observed between UC and SC subjects. The proportion of TGF- β ⁺ cells was similar among the groups in all of the T cell populations analyzed (CD4⁺, CD4⁺CD25⁺ and CD4⁺CD25⁻). This result suggests that the Tregs from CL patients exerted efficient suppressor activity due to increased production of IL-10.

4. Discussion

To clarify whether the lesion development in *L. braziliensis*-induced CL is related to Treg function impairment, we characterized the functional status of circulating Tregs in patients with active CL as well as in subjects with SC infection and UC. We first compared the frequencies of Tregs in the circulation of CL patients, UC and SC subjects, and we found similar frequencies of circulating Tregs among the groups, suggesting that the development of the disease was not associated with abnormalities in the circulating Treg frequencies. Conversely, during infection with *Trypanosoma cruzi* [13], *Toxoplasma gondii* [14] and in autoimmune diseases, [15–17] a decreased frequency of circulating Tregs is associated with the over-activation of T cells and tissue damage. Normal levels of circulating Tregs and normal expression of IL-10R on circulating CD4⁺ T lymphocytes did not explain the strong production of IFN- γ and TNF- α and lesion development in CL patients [1, 6]. It has been shown that decreased IL-10R expression is associated with exaggerated pro-inflammatory

responses in rheumatoid arthritis [18] because cells do not properly respond to IL-10-mediated suppression. However, altered IL-10R expression levels also did not support our first hypothesis.

Therefore, we next assessed the ability of CL patients Tregs to suppress T cell proliferation and pro-inflammatory cytokine production in total PBMC by comparing the responsiveness of CL patients' and UC PBMC to CL patients' and UC Treg suppressive mechanisms. The results showed that CL patients were not impaired in Treg function, and that their PBMC were responsive to Treg suppressive mechanisms. In fact, CL Tregs were more efficient at suppressing T cell proliferation and PBMC pro-inflammatory cytokine production than Tregs from healthy people, as was observed for TNF- α and IL-17 under polyclonal stimulus and for IFN- γ under SLA stimulus. The results were more evident in co-cultures where the Treg : PBMC ratio was 1 : 5. Due to the low number of CD4⁺CD25⁺ cells after purification, it was not possible to perform co-cultures with higher Treg : PBMC ratios, which we believe, based in our results, would probably show more evidently the higher efficacy of CL patients' Tregs. We have previously shown that CD4⁺CD25⁺ T cells from CL patients infected with *L. braziliensis* were able to suppress allogeneic lymphocyte proliferation in response to PHA [12]. However, we extended these observations by showing that Tregs from CL patients are more effective than UC Tregs at modulating PBMC activation.

An intense inflammatory reaction occurs in the ulcers of CL patients with strong IFN- γ production [19]. This compartmentalization of the immune response occurs during inflammatory diseases; therefore, it could be possible that the inflammatory cells present at the infection site are not responding properly to the immune modulation exerted by Tregs. It is difficult to predict what is the optimal Treg : effector cells ratio that are in close contact within an inflammatory infiltrate, which would allow to test Treg function in a physiological setting. Nevertheless, to answer our question, we cultured lesion infiltrating mononuclear cells in the presence of PBMC Tregs in the ratio that had retrieved us the most evident results previously, and we found that the inflammatory cells present in CL lesions also responded well to the suppressive mechanisms exerted by CL patients' Tregs. We have previously shown that the Tregs from lesions that were expanded *in vitro* were able to suppress lymphocyte proliferation [12]. In the present work, we did not identify which specific cell populations from the lesions had their cytokine production suppressed by Tregs. SLA stimulus may trigger pro-inflammatory production by T cells [20], but since it contains molecules that can be recognized by innate cell receptors such as Toll like receptors [21, 22], it may also have activated macrophages and dendritic cells, which can produce mainly TNF- α in *Leishmania* infection [23, 24]. However, it is known that Tregs have the capability to modulate the activity and cytokine production different leukocytes, as innate immune antigen presenting cells and CD8⁺ T cells, for example [9, 25]. Therefore, in the present work, we add that the inflammatory mononuclear cells from lesions of CL patients are responsive to Treg immune modulation.

Up to 16% of subjects living in *L. braziliensis* endemic areas have a positive DTH reaction to *Leishmania* antigens but do not have an active or past history of CL [26]. The individuals with SC *L. braziliensis* infection produce lower amounts of TNF- α and IFN- γ than the patients with CL [7, 27]. This could be due to increased function of Tregs, which could prevent lesion formation upon *L. braziliensis* infection. To determine whether this was the case, we compared the function of SC subjects' Tregs and CL patients' Tregs. We first analyzed PBMC, and found similar frequencies of circulating CD4⁺CD25^{high}FoxP3⁺ cells in SC subjects and CL patients, while the frequency of CD127⁻ cells among CD4⁺CD25^{high} cells was lower in SC subjects compared to CL patients. However, SC subjects' Tregs behaved similarly to CL patients' Tregs regarding their ability to suppress T cell proliferation and PBMC pro-inflammatory cytokine production. Since CD127 expression

was different between CD4⁺CD25^{high} cells from SC subjects and CL patients, it could be possible that a purification of Tregs based also in CD127 expression could lead to a different result. However, our data suggests that the difference in the expression of CD127 did not affect Treg function, since Tregs from both groups behaved similarly, even presenting distinct CD127 expression. In fact, it was shown in mice that differences in CD127 expression in FoxP3⁺ cells do not alter their suppressive capacity [28]. Despite no difference in suppression was observed, there was a higher percentage of Tregs in CL patients expressing IL-10 after stimulation. Therefore, these results suggested that the Tregs from SC subjects were less activated than those from CL patients. A recent study conducted in Colombia, however, showed that CL patients' Tregs suppressed IFN- γ production less efficiently than SC subjects' Tregs [29]. However, most of the patients in the study were infected by *L. panamensis*. Furthermore, the ability of SC Tregs to down modulate activity of CL patients' cells and vice-versa was not tested, and the patients with CL had active disease for at least 6 months. It is known that even parasites from the same species have genetic differences [30] and induce different degrees of inflammatory responses [31]. Therefore, considering distinct species, the differences in immune response may be still more evident. Regarding the duration of the illness, most of our patients presented with two months of active disease at most. Therefore, it is possible that patients with more than six months of disease represent a subgroup that has impaired Treg function because it has already been shown that pro-inflammatory responses are increased and overcome anti-inflammatory responses in the chronic form of the disease [32]. Interestingly, ML patients experience long-term persistence of the infection, which leads to the development of unresponsiveness by effector cells to regulatory mechanisms [19, 33, 34]. It will be important in future studies to analyze the function of CL patients' Tregs at different periods during the illness.

Finally, we found that the proportion of IL-10⁺ Tregs after stimulation of PBMC was higher in CL patients than in UC. Therefore, the increased efficacy in the suppression of T lymphocyte proliferation and PBMC pro-inflammatory cytokine production is likely due to the higher IL-10 secretion by CL patients Tregs because the addition of exogenous IL-10 to SLA-stimulated CL patients' PBMC cultures results in decreased IFN- γ production [33]. Moreover, the role of Tregs has been clearly shown in experimental murine leishmaniasis [10, 35, 36], as well as in human visceral leishmaniasis [37], where they act by suppressing Th1 responses and favoring parasite replication. Although Tregs have been shown to be able to produce IFN- γ in highly polarized Th1 environments, [38], and CL patients present high production of Th1 cytokines, we observed that the expression of IFN- γ by CD4⁺CD25⁺ cells from CL patients was very low or undetectable, while IL-10 was predominantly produced by these cells (result not shown).

Altogether, these observations suggest that patients with active CL due to *L. braziliensis* infection have increased Treg activation compared to UC and SC subjects. The higher Treg activity observed in CL patients compared to UC may be simply a consequence of the presence of the parasite, which is not found in UC. However, this may not be true for SC subjects, since it is unknown whether they carry latent infection, or clear it completely. Since few parasites are inoculated within a single sandfly bite [39], differences in parasite load in the onset of infection seem to be unlikely in determining which people will and which will not clear the infection or develop lesions. On the other hand, it may be possible that this increased activation of Tregs found in CL patients happen since the onset of infection, which this may be driven by genetic characteristic of the host, of the parasite strain, or both. As a consequence, higher Treg activation may favor parasite replication and persistence, generating delay in elimination of parasites and a chronic inflammatory response despite the presence of regulatory T cells, leading to lesion development, what may not occur in SC subjects. Supporting this hypothesis, a study that conducted genetic

polymorphism analysis of the IL-10 gene promoter in people with active 16 and subclinical *L. braziliensis* infection, showed that the presence of a specific allele that results in higher production of IL-10 is associated with development of the cutaneous lesions [40]. Therefore, the initial steps of infection, and Treg activity in this early phase, may play important role in the disease outcome, and studies addressing the first steps of *L. braziliensis* infection in humans are necessary, but are very difficult to conduct, since patients seek medical care when the lesions are already established to some time, and the real infection may have taken course long before lesion development.

Interestingly, IL-10⁺ cells in CL patients were more frequent not only in CD4⁺CD25⁺ population but also in the CD4⁺CD25⁻ one. There is a population of CD4⁺CD25⁻FoxP3⁻ cells that produces considerable amounts of IL-10 and exerts suppressor activity, which are the T regulatory type 1 (Tr-1) cells [41–44]. Cells with Tr-1 characteristics have been found in experimental infections with *Leishmania*, and they produce high levels of IL-10, resulting in the suppression of effector cells [45–47]. Tr-1 cells have been suggested to be the main source of IL-10 during human visceral leishmaniasis [48, 49]. It is possible, therefore, that the IL-10 produced in CD4⁺CD25⁻ cells in the *L. braziliensis*-infected CL patients are derived from Tr-1 cells or Tr-1-like cells. As such, these cells could, in conjunction with Tregs, also contribute to the suppression of effector cell function and favor parasite survival.

Our study brings new light to the mechanisms by which *L. braziliensis* may exploit the regulatory immune system upon infection. Immunological strategies that interfere with these parasite escape mechanisms may improve CL treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CL	cutaneous leishmaniasis
IL-10R	IL-10 receptor
ML	mucosal leishmaniasis
SC	sub-clinical
SLA	soluble <i>Leishmania</i> antigen
Tr-1	T regulatory type 1
Treg	regulatory T cell
UC	uninfected controls

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Figure 1.

CD4⁺ and CD4⁺CD25⁺ T cell immunophenotyping. The graphs show samples of PBMC from CL patients (black bars, n = 21), UC (white bars, n = 18) and SC subjects (gray bars, n = 5). (A) Percentages of CD4⁺CD25⁺, CD4⁺CD25^{high}, CD4⁺IL-10R⁺ (CD210) and CD4⁺CD25⁻ T cells inside the lymphocyte gate. (B) Percentage of FoxP3⁺, CCR4⁺ and CD127⁻ cells inside the CD4⁺CD25^{high} gate. The results are expressed as the mean $\pm$ standard error of the cell percentage. Statistical analysis: Non-parametric Mann-Whitney test. * $p < 0.05$.

Figure 2.

Lymphocyte proliferation suppression assay. $CD4^+CD25^+$ cells isolated from CL patients and UC PBMC were cultured in the presence of CL patients or UC total PBMC and stimulated with anti-human $CD3\epsilon$ chain monoclonal antibody ($2.5 \mu\text{g/ml}$). The cells were cultured at a ratio of 1 $CD4^+CD25^+$ cell to 10 or 5 total PBMC for 96 hours. The data are shown as the percentage of proliferating cells. (A) CL patients PBMC co-cultured with CL patients $CD4^+CD25^+$ cells; (B) UC PBMC co-cultured with CL patients $CD4^+CD25^+$ cells; (C) CL patients PBMC co-cultured with UC $CD4^+CD25^+$ cells; (D) UC PBMC co-cultured with UC $CD4^+CD25^+$ cells. Cell proliferation in non-stimulated cultures was below detection thresholds. Statistical analysis: two-tailed Wilcoxon matched-pairs signed rank test ($n = 13$ paired samples). * $p < 0.05$ in relation to No Treg cultures.

Figure 3.

Cytokine production suppression assay under polyclonal stimulus. $CD4^+CD25^+$ cells isolated from CL patients or UC PBMC were cultured in the presence of UC (white symbols) or CL patients (black symbols) total PBMC in the proportion of 1 $CD4^+CD25^+$ cell to 10 or 5 total PBMC, and stimulated with anti-human $CD3\epsilon$ chain monoclonal antibody (2.5 $\mu\text{g/ml}$) for 72 hours. (A, B and C): Concentrations of cytokines in the culture supernatants. (A): IFN- γ ; (B): TNF- α ; (C): IL-17. (D, E and F): Comparison of the suppressive activity of $CD4^+CD25^+$ cells from CL patients and UC specimens. at the ratio of 1 $CD4^+CD25^+$ cell to 5 total PBMC. The results show the percentages of suppression of the production of IFN- γ (D), TNF- α (E) and IL-17 (F). (G, H and I): Comparison of the susceptibility of UC (white bars) and CL patients (black bars) PBMC to $CD4^+CD25^+$ cell-mediated suppression. The cells were cultured at a ratio of 1 $CD4^+CD25^+$ cell to 10 or 5 total PBMC. The results show means $\pm$ standard errors of the suppression of the production of IFN- γ (G), TNF- α (H) and IL-17 (I). Cytokine concentrations in non-stimulated cultures supernatants were below detection thresholds. Statistical analysis: two-tailed Wilcoxon matched-pairs signed rank test ($n = 14$ paired samples for A, D and G; $n = 15$ paired samples for B, E and H; $n = 11$ paired samples for C, F and I). * $p < 0.05$.

Figure 4.

CD4⁺CD25⁺ cells isolated from CL patients and UC PBMC were cultured in the presence of CL patients total PBMC and stimulated with (SLA) (5 µg/ml). The cells were cultured at a ratio of 1 CD4⁺CD25⁺ cell to 5 total PBMC for 72 hours. The data are shown as the percentage of suppression of IFN-γ (A), TNF-α (B) and IL-17 (C) production. Cytokine concentrations in non-stimulated cultures supernatants were below detection thresholds. Statistical analysis: two-tailed Wilcoxon matched-pairs signed rank test (n = 12 paired samples for A; n = 11 paired samples for B; n = 10 paired samples for C). * *p* < 0.05.

Figure 5.

Lymphocyte proliferation and cytokine production suppression assays with samples from CL patients and SC subjects. $CD4^+CD25^+$ cells isolated from CL patients and SC subjects PBMC were cultured in the presence of CL patients or SC subjects total PBMC and stimulated with anti-human CD3 ϵ chain monoclonal antibody (2.5 μ g/ml). The cells were cultured at a ratio of 1 $CD4^+CD25^+$ cell to 10 or 5 total PBMC for 72 hours for cytokine quantification and 96 hours for lymphocyte proliferation assays. The data are shown as the percentage of proliferating cells (A) and the concentration of IFN- γ (B) and TNF- α (C) in the culture supernatants. Cytokine concentrations in non-stimulated cultures supernatants were below detection thresholds. Statistical analysis: two-tailed Wilcoxon matched-pairs signed rank test (n = 4 paired samples for A; n = 5 paired samples for B and C).

Figure 6.

Cytokine production suppression assay in lesion infiltrating mononuclear cells. The CL patients lesion infiltrating mononuclear cells stimulated with soluble *Leishmania* antigen (SLA) (5 µg/ml) were cultured in the absence (white bars) or presence (black bars) of CD4⁺CD25⁺ cells isolated from CL patients PBMC. The cells were cultured at a ratio of 1 CD4⁺CD25⁺ cell to 5 total lesion infiltrating mononuclear cells for 72 hours. The data are shown as the concentration of IFN-γ (A) and TNF-α (B) in the culture supernatants. Cytokine concentrations in non-stimulated cultures supernatants were below detection thresholds. Statistical analysis: two-tailed Wilcoxon matched-pairs signed rank test (n = 9 for A and B). **p* < 0.05.

Figure 7.

Intracellular cytokine staining. The graphs show samples of PBMC from CL patients (black bars, $n = 12$), UC (white bars, $n = 6$), and SC subjects (gray bars, $n = 5$). Shown are the percentages of IL-10⁺ and TGF-β⁺ CD4⁺ T cells (A), IL-10⁺ and TGF-β⁺ CD4⁺CD25⁺ cells (B), and IL-10⁺ and TGF-β⁺ CD4⁺CD25⁻ cells (C). The results are expressed as the means $\pm$ standard errors of the percentage of cells. Statistical Analysis: Non-parametric Mann-Whitney test. * $p < 0.05$.

Table 1

Clinical features of the 45 patients with cutaneous leishmaniasis and 6 subjects with subclinical infection participating of the study.

Variables	CL patients		SC subjects	
	Mean + SD	Range	Mean + SD	Range
Age	31.5 ± 13.4	8 to 67	44.5 ± 20.3	19 to 75
Gender M:F	73.3 : 26.7*	NA	40 : 60*	NA
Illness duration (days)	40 ± 31.7	14 to 150	NA	NA
Numbers of lesions	1.4 ± 0.7	1 to 4	NA	NA
Largest lesion (mm ²)	470.3 ± 722.7	40 to 3,600	NA	NA
Leishmania skin test (mm ²)	179.1 ± 89.2	25 to 400	256.7 ± 202.3	42 to 550

* Proportion

NA = Not applicable

Table 2

Lymphocyte proliferation suppression assay.

	Suppression of proliferation (%)			
	1 : 10 ^{a)}		1 : 5 ^{a)}	
	CL PBMC	UC PBMC	CL PBMC	UC PBMC
CL CD4 ⁺ CD25 ⁺	60.43 (±23.40)	52.08 (±25.31) <i>p</i> =0.5417 ^{c)}	72.51 (±23.34)	71.91 (±17.30) <i>p</i> =0.7869 ^{c)}
UC CD4 ⁺ CD25 ⁺	50.85 (±26.55) <i>p</i> =0.0327 ^{b)}	54.27 (±17.73) <i>p</i> =0.6848 ^{b)}	70.10 (±23.42) <i>p</i> =0.6848 ^{b)}	70.00 (±19.11) <i>p</i> =0.9697 ^{b)}

^{a)} CD4⁺CD25⁺ cells isolated from CL patients and UC PBMC were cultured in the presence of CL patients or UC total PBMC and stimulated with anti-human CD3ε chain monoclonal antibody (2.5 µg/ml). The cells were cultured at a ratio of 1 CD4⁺CD25⁺ cell to 10 or 5 total PBMC.

^{b)} Vertical *p* values are related to the comparison of the percentages of suppression induced by CD4⁺CD25⁺ cells from each group.

^{c)} Horizontal *p* values are related to the comparisons of the percentages of suppression induced in the PBMC from each group.

b) and c) Statistical analysis: two-tailed Wilcoxon matched-pairs signed rank test.