# Tr-1–Like $CD4^{+}CD25^{-}CD127^{-/low}FOXP3^{-}$ Cells Are the Main Source of Interleukin 10 in Patients With Cutaneous Leishmaniasis Due to *Leishmania braziliensis*

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**$CD4^{+}CD25^{+}FOXP3^{+}$ regulatory T cells have long been shown to mediate susceptibility to *Leishmania* infection, mainly via interleukin 10 production. In this work, we showed that the main sources of interleukin 10 in peripheral blood mononuclear cells (PBMCs) from patients with cutaneous leishmaniasis due to *Leishmania braziliensis* are $CD4^{+}CD25^{-}CD127^{-/low}FOXP3^{-}$ cells. Compared with uninfected controls, patients with CL had increased frequencies of circulating interleukin 10–producing $CD4^{+}CD25^{-}CD127^{-/low}$ cells, which efficiently suppressed tumor necrosis factor α production by the total PBMC population. Also, in CL lesions, interleukin 10 was mainly produced by $CD4^{+}CD25^{-}$ cells, and interleukin 10 messenger RNA expression was associated with interleukin 27, interleukin 21, and interferon γ expression, rather than with FOXP3 or transforming growth factor β expressions. Active production of both interleukin 27 and interleukin 21, together with production of interferon γ and interleukin 10, was also detected in the lesions. Since these cytokines are associated with the differentiation and activity of Tr-1 cells, our results suggest that this cell population may play an important role in the immunomodulation of CL. Therefore, development of treatments that interfere with this pathway may lead to faster parasite elimination.**

***Keywords.*** *L. braziliensis*; tegumentary leishmaniasis; Tr-1 cells; IL-10.

Upon entrance to the vertebrate host, parasites from the *Leishmania* genus infect phagocytic cells, in which they are able to multiply and establish infection [1]. Mouse studies have shown that the development of protective T-helper type 1 (Th1) responses and production of interferon γ (IFN-γ) result in the activation of microbicidal mechanisms in infected cells and parasite killing [2–4]. The importance of Th1 responses for parasite killing has also been shown in humans [5–7]. In experimental models, the generation of Th2 responses, characterized by interleukin 4 (IL-4) production, has long been considered the main factor promoting the survival of *Leishmania* parasites in the host [2, 8, 9]. More recently, however, regulatory T cells (Tregs) have also been shown to be important mediators of susceptibility to *Leishmania* infection [10, 11]. These cells have the $CD4^{+}CD25^{high}FOXP3^{+}$ phenotype [12] and mediate their suppressive activity by several mechanisms, such as the production of cytokines, mainly interleukin 10 (IL-10) [13, 14] and transforming growth factor β (TGF-β) [15], which directly suppress effector T-cell activity; contact-dependent mechanisms that act on antigen-presenting cells (APCs) through surface molecules, such as CTLA-4 [16], decreasing the ability

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of APCs to activate effector T cells; and sequestration of interleukin 2 (IL-2) via CD25 (IL-2Rα) [17], which is highly expressed in Tregs [18], resulting in impaired effector T-cell proliferation. Tregs have been shown to accumulate in lesions of *Leishmania major*–infected mice [19, 20] and are able to suppress the activities of Th1 effector cells, leading to impaired parasite elimination [21].

*Leishmania braziliensis* is one of the main causative agents of localized cutaneous leishmaniasis (CL) and mucosal leishmaniasis (ML) in the Americas, mainly in Brazil [22]. Although the specific role of Tregs in *L. braziliensis* infection has not been characterized, our group showed that these cells accumulate in the lesions of *L. braziliensis*–infected patients [23], who demonstrate increased Treg activation, compared with healthy controls, a factor that may contribute to impaired parasite elimination [24].

There is also a population of induced regulatory T cells, named T-regulatory type 1 (Tr-1) cells, which have the $CD4^+CD25^-FOXP3^-$ phenotype and produce high levels of IL-10 [25, 26]. The production of interleukin 27 (IL-27) by APCs activates the expression of the transcription factor c-Maf in $CD4^+$ T cells. Subsequently, c-Maf induces the production of IL-10 and interleukin 21 (IL-21), working as an autocrine factor to sustain the production of IL-10, thereby stimulating the development of Tr-1 cells [27, 28]. Regarding the origin of IL-10 in *Leishmania* infections, it was recently suggested that although natural Tregs contribute to IL-10 production, the main source of this cytokine in *L. major*–infected mice is $CD4^+CD25^-FoxP3^-$ cells. Also, these cells are the main mediators of immunosuppression during infection [29]. In visceral leishmaniasis, where IL-10 is the most important suppressor cytokine produced [30], $CD4^+CD25^-FoxP3^-$ Tr-1–like cells are the main source of this cytokine in humans and mice [31, 32]. We have previously shown that $CD4^+CD25^-$ cells contribute to IL-10 production in patients with CL [24]. However, these cells have never been characterized for their potential Tr-1 characteristics or for their role in human cutaneous leishmaniasis due to *L. braziliensis*. In the present work, we show for the first time that $CD4^+CD25^-CD127^{-/low}$ cells are the main source of IL-10 in the circulation of patients with CL and that they suppress tumor necrosis factor α (TNF-α) production by peripheral blood mononuclear cells (PBMCs). Moreover, we show that in lesions of patients with CL, the expression of IL-10 is correlated with IFN-γ, IL-27, and IL-21, rather than with FOXP3. Actually, the majority of IL-10 is produced by $CD4^+CD25^-$ cells. Therefore, this work provides new insight regarding the mechanisms by which parasites may modulate T-cell–specific responses to survive in the host.

## MATERIALS AND METHODS

### Patients With CL and Uninfected Controls

PBMCs and skin biopsy specimens were obtained from patients with CL admitted to the health post of Corte de Pedra, Bahia, Brazil. All individuals received a diagnosis of *L. braziliensis* infection according to the presence of a typical CL lesion, a positive result of a *Leishmania* skin test (induration, ≥5 mm), and parasite isolation or detection of parasite DNA by polymerase chain reaction (PCR) [33]. Information from patients with CL is summarized in Table 1. As controls, we used PBMCs from 16 uninfected donors, including 8 men and 8 women, ranging from 22 to 47 years of age, and biopsy specimens from uninfected controls with rejection of plastic surgery (4 samples). Informed consent was obtained from all participants, and all procedures were approved by the local ethics committees from the University Hospital Complex Edgar Santos, Federal University of Bahia.

### PBMC Isolation and Flow Cytometry

PBMCs from patients with CL and uninfected controls were isolated through a Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden) gradient and stained for CD4-FITC, CD25-PECy7, CD127-Alexa Fluor 750, FOXP3-PE, or IL-10-PE (BD and eBiosciences, San Diego, CA). For intracellular cytokine detection, PBMCs were stimulated with 5 µg/mL of soluble *Leishmania* antigen (SLA) and cultured for 72 hours at 37°C in 5% $CO_2$. Next, supernatants were removed, and cells were stimulated with PMA (10 ng/mL), ionomycin (500 ng/mL), and brefeldin for 6 hours before staining. The samples were acquired with a FACSCanto II flow cytometer (BD Biosciences), and analyses were performed using FACSDiva software (BD Biosciences) and FlowJo software (TreeStar, Ashland, OR).

### Isolation of $CD4^+CD25^-CD127^{-/low}$ Cells and Co-cultures

$CD4^+CD25^-CD127^{-/low}$ cells were purified from PBMCs from patients with CL, using the $CD4^+CD25^+CD127^{dim/-}$ Regulatory T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). Next, unsorted PBMCs from patients with CL and uninfected controls were stained with CFSE and cocultured with purified $CD4^+CD25^-CD127^{-/low}$ cells at ratios of $CD4^+CD25^-CD127^{-/low}$ cells to PBMCs of 1:5 and 1:10, resulting in $2 \times 10^5$ cells per well, which were stimulated with anti-human CD3ε (2.5 µg/mL; BD Biosciences) or SLA (5 µg/mL). After 72 hours, TNF-α and IFN-γ levels were quantified in culture supernatants by enzyme-linked immunosorbent assay

**Table 1. Demographic and Clinical Characteristics of 34 Patients With Cutaneous Leishmaniasis**

| Variables | Mean ± SD | Range |
|---|---|---|
| Age, y | 28.6 ± 11.9 | 4–62 |
| Illness duration, d | 31.6 ± 13.8 | 15–60 |
| Lesions, no. | 1.4 ± 0.7 | 1–4 |
| Largest lesion area, mm$^2$ | 262.5 ± 412.5 | 35–2400 |
| *Leishmania* skin test induration, mm$^2$ | 224.2 ± 115.8 | 36–506 |

Eighteen subjects (52.9%) were male, and 16 (47.1%) were female.

(BD Biosciences). Alternatively, cells were cultured for 96 hours, and T-cell proliferation was evaluated by CFSE dilution, using a FACSCanto II flow cytometer (BD Biosciences) and FlowJo software (TreeStar). All cell culture procedures were performed at 37°C and 5% $CO_2$.

### Immunohistochemistry and Confocal Microscopy

Biopsy specimens from lesion borders of patients with CL were frozen in optimal-cutting-temperature compound (Sakura Finetek, Torrance, CA), sectioned, and fixed in ice-cold acetone before incubation with anti-human IFN-γ, IL-10, IL-27, and IL-21 antibodies (Abcam, Cambridge, MA; Santa Cruz Biotechnologies, Santa Cruz, CA). The avidin-biotin peroxidase method, followed by incubation with 3,30-diaminobenzidine, was used for detecting cytokine-producing cells. Photomicrographs were collected in a light microscope. For confocal microscopy, samples were incubated with anti-human CD4, CD25, and IL-10 antibodies (Abcam and Santa Cruz). Secondary antibodies conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, CA) were used for CD4 and CD25 staining. For IL-10 detection, samples were incubated with a secondary biotinylated antibody (Vector, Burlingame, CA), followed by streptavidin conjugated to Alexa Fluor 647 (Invitrogen). Prolong Gold antifade reagent with DAPI (Invitrogen) was used for placing coverslips. Photomicrographs were created using Leica SP2 and SP5 confocal microscopes (Leica Microsystems, Wetzlar, Germany).

### Real-Time PCR

RNA was extracted from biopsy specimens from patients with CL by using Trizol reagent (Invitrogen) and RNA extraction columns (GE Healthcare). Complementary DNA was synthesized using 1 µg of RNA through a reverse transcription reaction. For real-time PCR, SYBR Green and StepOnePlus Real-Time PCR systems (Applied Biosystems, Warrington, United Kingdom) were used, and expression of each gene was normalized to GAPDH expression in the same samples. The relative expression of each gene in samples from patients with CL was calculated in relation to that observed in healthy skin samples. Gene expression was calculated using the equation $2^{-\Delta\Delta Ct}$, calculated as follows: ΔΔCt = [CL sample–specific mRNA Ct – CL sample GAPDH mRNA Ct] – [UC-specific mRNA Ct – UC GAPDH mRNA Ct], with "Ct" denoting the cycle threshold and "mRNA" denoting messenger RNA. The primers used in this study are shown in Table 2.

### Statistical Analyses

Statistical analyses were performed using GraphPad prism 5.0 software (GraphPad Software, San Diego, CA). The tests used were as follows: 2-tailed nonparametric Mann–Whitney test, 2-tailed Wilcoxon matched-pairs signed rank test, and linear regression followed by the Spearman correlation test. *P* values of ≤ .05 were considered significant for all analyses.

## RESULTS

### $CD4^+CD25^-CD127^{-/low}$ Cells Are the Main Sources of IL-10 in the Circulation of Patients With CL

In a previous study, we demonstrated that $CD4^+CD25^-$ cells participate in the production of IL-10 by $CD4^+$ T cells among PBMCs from patients with CL [24], although we had yet to show which population of $CD4^+$ T cells contributes most significantly to IL-10 production. To answer this question, we first analyzed the expression of CD25 in IL-10–producing, SLA-stimulated, and PMA-ionomycin–activated $CD4^+$ cells from patients with CL and uninfected controls and observed that the $CD25^-$ cells were the main source of IL-10 in both groups (Figure 1*A* and 1*D*). In humans, IL-10–producing Tr-1 cells are often $CD25^-CD127^{-/low}$ and $FOXP3^-$. Moreover, these cells may produce IFN-γ concomitantly with IL-10 [34]. Therefore, we characterized the IL-10–producing $CD4^+CD25^-$ cells in terms of their CD127 expression and observed that, in patients with CL and uninfected controls, most of the cells were $CD127^{-/low}$ (Figure 1*B* and 1*D*) and that, among the peripheral blood $CD4^+CD25^-CD127^{-/low}$ cells, most were $FOXP3^-$ in both patients with CL and uninfected controls (Figure 1*C*). Additionally, we observed a similar pattern of IFN-γ production among IL-10–producing $CD4^+CD25^-CD127^{-/low}$ cells in both groups (Figure 1*E* and 1*F*). These results show that the main sources of IL-10 among activated PBMCs from uninfected

**Table 2. Real-Time Polymerase Chain Reaction Primer Sequences**

| Target Gene | Sense Sequence | Antisense Sequence |
|---|---|---|
| *IFNG* | 5′-GAG AAC CCA AAA CGA TGC A-3′ | 5′-ACT TCT TTG GCT TAA TTC TCT CG-3′ |
| *IL10* | 5′-AGA TCT CCG AGA TGC CTT CA-3′ | 5′-ATT CTT CAC CTG CTC CAC GG-3′ |
| *TGFB1* | 5′-ATT GAG GGC TTT CGC CTT AG-3′ | 5′-TGT GTT ATC CCT GCT GTC ACA-3′ |
| *FOXP3* | 5′-CCC ACT TAC AGG CAC TCC TC-3′ | 5′-CTT CTC CTT CTC CAG CAC CA-3′ |
| *IL27p28* | 5′-CCA GTA ACT GAA AGC CCC TCT-3′ | 5′-AAC CAT CAT CTC CCT AAA CAA TAA A-3′ |
| *IL21* | 5′-CCA CAA ATG CAG GGA GAA GA-3′ | 5′-GAA TCA CAT GAA GGG CAT GTT-3′ |
| *GAPDH* | 5′-CAT CAA TGA CCC CTT CAT TGA-3′ | 5′-GAA TTT GCC ATG GGT GGA-3′ |

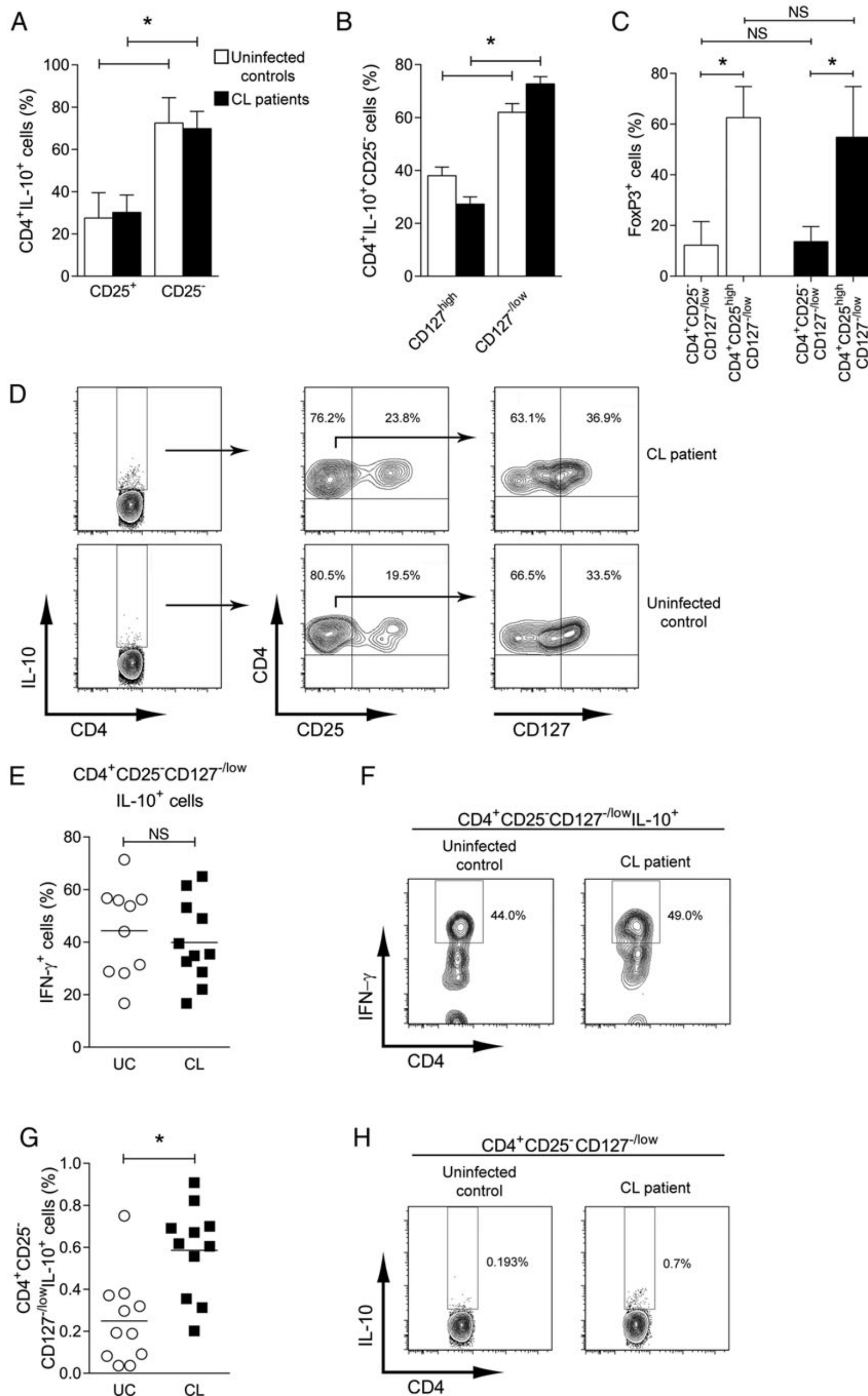


**Figure 1.** Identification of cellular sources of interleukin 10 (IL-10) in peripheral blood mononuclear cells (PBMCs). Graphs show samples of PBMCs from 11 patients with cutaneous leishmaniasis (CL; black bars) and 11 uninfected controls (white bars). Total PBMCs were freshly stained (C) or cultured in the presence of 5 µg/mL of soluble *Leishmania* antigen (SLA) for 72 hours, and for additional 6 hours in the presence of PMA (10 ng/mL), ionomycin (500 ng/mL), and brefeldin before staining (*A*, *B*, *D*, *E*, *F*, *G*, and *H*). *A*, Percentages of CD25$^+$ and CD25$^−$ cells among CD4$^+$IL-10$^+$ cells. *B*, Percentages of CD127$^{high}$ and CD127$^{−/low}$ cells among CD4$^+$CD25$^−$IL-10$^+$ cells. *C*, Percentages of FOXP3$^+$ cells among CD4$^+$CD25$^{high}$CD127$^{−/low}$ and CD4$^+$CD25$^−$CD127$^{−/low}$ cells. *D*, Representative dot plots of graphs *A*, *B*, and *C*. *E*, Percentages of interferon γ (IFN-γ)–expressing cells among CD4$^+$CD25$^−$CD127$^{−/low}$IL-10$^+$ cells. *F*, Representative dot plot of data in panel *E*. *G*, Percentages of IL-10–producing CD4$^+$CD25$^−$CD127$^{−/low}$ cells. *H*, Representative dot plot of data in panel *E*. Results are represented as means ± standard errors of means (*A*–*C*) or as individual values plus mean (*E* and *G*). **P* < .05 or not significant (NS), by the nonparametric Mann–Whitney test.

controls and patients with CL are Tr-1 like $CD4^+CD25^-$ $CD127^{-/low}$ cells that do not express FOXP3. Finally, we compared the frequency of IL-10–producing $CD4^+CD25^-CD127^{-/low}$ cells in patients with CL and uninfected controls and found that the patients with CL had a higher frequency of $CD4^+CD25^-CD127^{-/low}IL\text{-}10^+$ cells in the circulation than the uninfected controls (Figure 1*G* and 1*H*). Moreover, IL-10 production by $CD4^+CD25^-CD127^{-/low}$ cells from uninfected controls and patients with CL was similar upon stimulation with medium alone or tetanus toxoid before activation by PMA and ionomycin (Supplementary Figure 1A–C). Taken together, these results show that, among PBMCs from *L. braziliensis*–infected patients with CL, antigen-specific Tr-1–like cells are the most important source of IL-10.

### $CD4^+CD25^-CD127^{-/low}$ Cells From Patients With CL Efficiently Suppress TNF-α Production but Not T-Lymphocyte Proliferation or IFN-γ Production

We next sought to determine whether $CD4^+CD25^-CD127^{-/low}$ cells from patients with CL had suppressive capabilities. We isolated these cells and cultured them in the presence of total PBMCs from patients with CL or uninfected controls at $CD4^+CD25^-CD127^{-/low}$ cell to total PBMC ratios of 1:10 or 1:5. These cells were not able to efficiently suppress T-cell proliferation in the PBMCs from patients with CL or uninfected controls at the described coculture ratios. However, in anti-human CD3ε (2.5 µg/mL)–stimulated cultures, when $CD4^+$ $CD25^-CD127^{-/low}$ cells from patients with CL were cocultured at ratios of 1:5 and 1:10 with PBMCs from either group, the production of TNF-α was significantly lower than in the absence of purified $CD4^+CD25^-CD127^{-/low}$ cells (Figure 2*A*). When PBMCs from patients with CL were stimulated with *Leishmania* antigen (SLA), TNF-α concentrations in the supernatants of cocultures diluted at $CD4^+CD25^-CD127^{-/low}$ cell to PBMC ratios of 1:10 and 1:5 were lower than that for PBMCs cultured alone, while the levels of TNF-α produced by PBMCs from uninfected controls upon stimulation with SLA were very low; therefore, it was not possible to evaluate the suppression of TNF-α production in this group (Figure 2*B*). The concentrations of IFN-γ in anti-CD3–stimulated cultures, however, did not decrease significantly in the presence of $CD4^+CD25^-CD127^{-/low}$ cells from patients with CL (Figure 2*C*). This observation is consistent

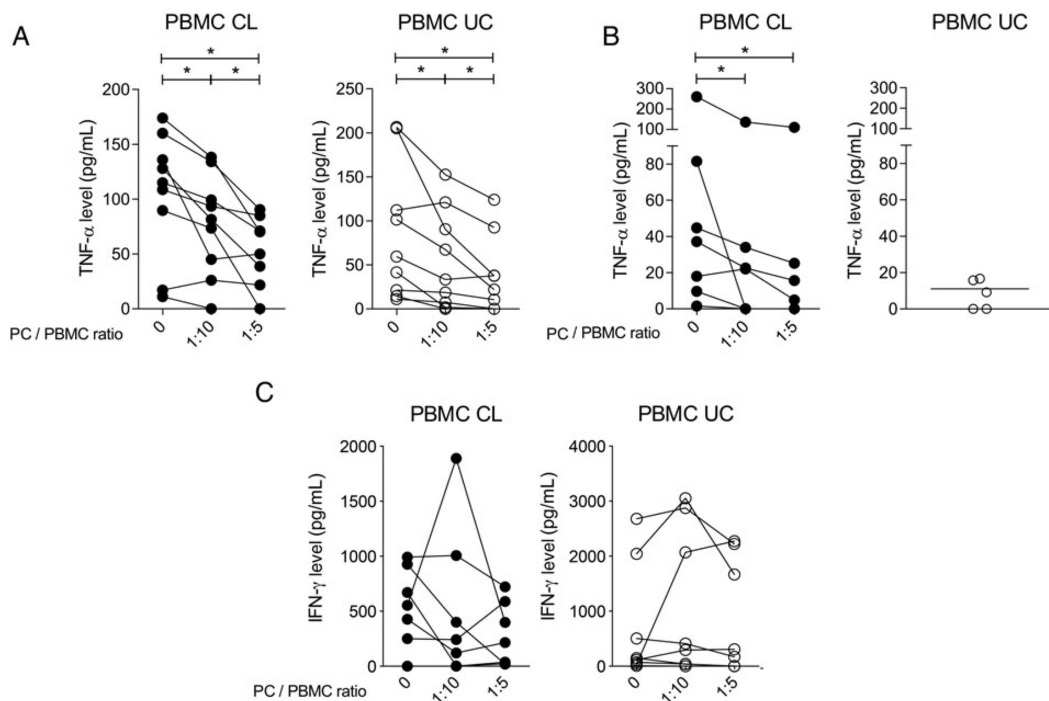


**Figure 2.** Tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ) production suppression assays. $CD4^+CD25^-CD127^{-/low}$ cells isolated from the peripheral blood mononuclear cells (PMBCs) of patients with cutaneous leishmaniasis (CL) were cultured in the presence of total PBMCs from patients with CL or uninfected controls (UC) stimulated with an anti-human CD3ε chain monoclonal antibody (2.5 µg/mL) or soluble *Leishmania* antigen (SLA; 5 µg/mL). Cells were cultured at ratios of 1 $CD4^+CD25^-CD127^{-/low}$ purified cell (PC) to 10 or 5 total PBMCs for 72 hours for TNF-α and IFN-γ quantification. *A*, Concentration of TNF-α in the supernatants of cocultures stimulated with 2.5 µg/mL of anti-CD3. *B*, Concentration of TNF-α in the supernatants of cocultures stimulated with 5 µg/mL of SLA. *C*, Concentration of IFN-γ in the supernatants of cocultures stimulated with 2.5 µg/mL of anti-CD3. **P* < .05, by the 2-tailed Wilcoxon matched-pairs signed rank test (n = 9 paired samples for panel *A*, n = 7 paired samples for panel *B*, and n = 7 paired samples for panel *C*).

with the fact that Tr-1 cells may also produce IFN-γ, in addition to IL-10 [34]. These results show the suppressor capacity of $CD4^+CD25^-CD127^{-/low}$ over the production of TNF-α by PBMCs from patients with CL and suggest that these cells may play an important role in the suppression of the immune response during *L. braziliensis* infection, since this cytokine plays an important role in parasite killing and resistance to infection [4, 35].

#### IL-10 mRNA Expression in the Lesions of Patients With CL Is Not Associated With FOXP3

We next evaluated the mRNA expression for IL-10 in the lesions of patients with CL and characterized other cytokines that could be associated with IL-10 production. For this, we obtained lesion biopsy specimens from patients with CL, quantified the mRNA expression of different cytokines and transcription factors known to be associated with IL-10 production, and identified correlations between these factors. Interestingly, we first observed that mRNA expression for IL-10 in the lesions was not directly or significantly correlated with the mRNA expression for either TGF-β (Figure 3*A*) or FOXP3 (Figure 3*B*). However, mRNA expression for IL-10 was directly and significantly correlated with the mRNA expression for IFN-γ (Figure 1*C*), IL-27 (Figure 1*D*), and IL-21 (Figure 1*E*). The expression levels for TGF-β and FOXP3 were directly and significantly correlated with one another (Figure 1*F*) but were not significantly correlated with the expression for IFN-γ (Figure 3*G* and 3*J*), IL-27 (Figure 3*H* and 3*K*), or IL-21 (Figure 3*I* and 3*L*). We also observed that mRNA expression for IFN-γ was directly and significantly correlated with that for IL-27 (Figure 3*M*) and IL-21 (Figure 3*N*) and that the expression levels for IL-27 and IL-21 were directly and significantly correlated with one another (Figure 3*O*). IL-10 may also be produced by Th2 cells, and therefore, we analyzed the participation of Th2 responses in the production of IL-10 by measuring mRNA expression for GATA-3, IL-4, and IL-5 in the same lesion samples. The results showed that in all cases, expression for GATA-3, IL-4 and IL-5 in lesions of patients with CL were lower than in healthy skin (Supplementary Figure 2*A*–*C*), ruling out the participation of Th2 cells in IL-10 production.

To confirm that the cytokine mRNA detected was actually translated into protein, we also performed immunohistochemistry analysis of frozen lesion sections from patients with CL and were able to detect IFN-γ (Figure 4*A*), IL-10 (Figure 4*B*), IL-27 (Figure 4*C*), and IL-21 (Figure 4*D*). Therefore, since IL-10 expression is associated with IL-27, IL-21, and IFN-γ expression, which are linked to Tr-1–cell differentiation, maintenance, and activity [28, 34], but is not associated with FOXP3 or TGF-β expression, which are linked to natural Tregs [36], these findings suggest that the IL-10 in the lesions of patients with CL is produced mainly by Tr-1–like cells, not by natural $FOXP3^+$ Treg cells.

#### $CD4^+CD25^-$ Cells Are the Major Producers of IL-10 in Lesions of Patients With CL

To confirm that natural Tregs were not the major source of IL-10 in the lesions of patients with CL, we performed immunofluorescence staining for CD4, CD25, and IL-10 in frozen sections of biopsy specimens from patients with CL. We detected IL-10 in regions containing inflammatory infiltrates throughout the lesions. When we stained for IL-10 and CD4, we observed that the vast majority of cells producing IL-10 also expressed CD4 (Figure 5*A*). However, when we stained for IL-10 and CD25, only a small fraction of the cells that produced IL-10 also expressed CD25 on their surface, while the majority of IL-10–producing cells did not express CD25 (Figure 5*B*). These results demonstrate that $CD4^+CD25^-$ cells are the major source of the IL-10 that is produced in the lesions of patients with CL.

## DISCUSSION

In this work, we characterized a population of IL-10–producing Tr-1–like cells that were capable of effectively suppressing the production of the proinflammatory cytokine TNF-α in patients with CL. First, we observed that, in the circulation of patients with CL, the main source of IL-10 was not natural $CD4^+CD25^+FOXP3^+$ regulatory T cells, but rather was $CD4^+CD25^-CD127^{-/low}$ cells, the majority of which were $FOXP3^-$. Although $CD4^+CD25^-CD127^{-/low}$ cells were also the main source of IL-10 in the circulation of uninfected controls, patients with CL presented increased frequencies of IL-10–producing $CD4^+CD25^-CD127^{-/low}$ cells, demonstrating that *L. braziliensis* infection induces IL-10 production by this cell population. Also, the fact that this difference was observed only upon SLA stimulation suggests that this IL-10–producing Tr-1–like population found in patients with CL is specific for *L. braziliensis* antigens. In fact, previous studies of experimental murine *L. major* infections identified $CD4^+CD25^-FoxP3^-$ cells as the main source of IL-10 production and found that these cells were capable of suppressing Th1 responses [20, 29, 37]. In human infections, $CD4^+CD25^-FOXP3^-$ cells produce IL-10 in visceral disease [30, 31], but its role is unknown in human cutaneous leishmaniasis. By showing that these cells express $CD127^{-/low}$ phenotype and by using this feature to isolate and to test their suppressive capacity, we suggest a new marker for nonnatural Tregs in cutaneous leishmaniasis. Also, using this approach we showed that these cells suppress TNF-α production by PBMCs from patients with CL.

The phenotype of this IL-10–producing cell population that we showed to be increased in patients with CL, compared with uninfected controls, is very similar to that of Tr-1 cells, which is $CD4^+CD25^-FOXP3^-CD127^{-/low}IL\text{-}10^+$ and may produce IL-10 and IFN-γ simultaneously [25, 34], which may explain why these cells did not suppress IFN-γ production significantly

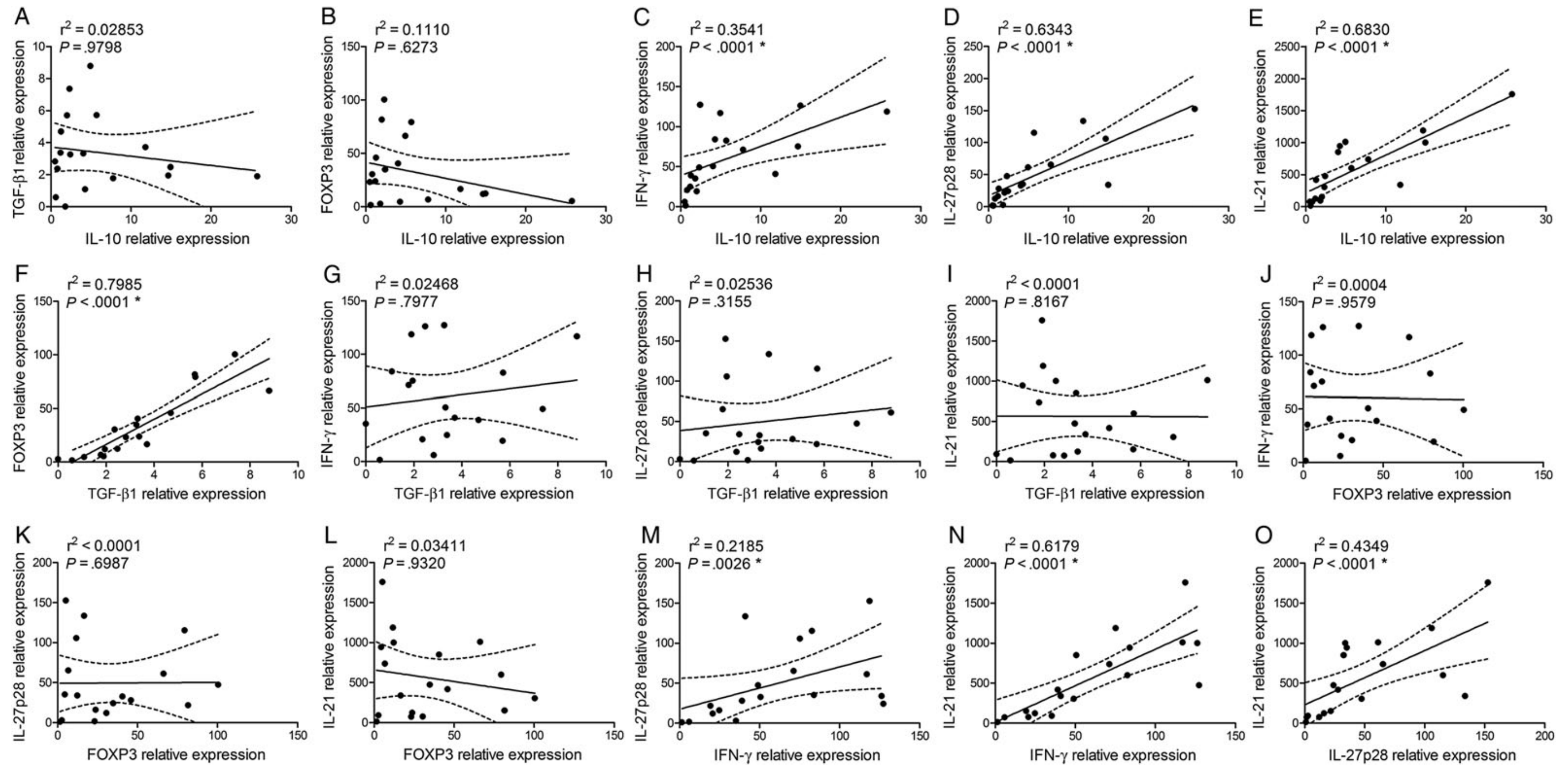


**Figure 3.** Correlations among messenger RNA (mRNA) expression levels for cytokines and FOXP3 in lesions of patients with cutaneous leishmaniasis. Graphs show relative mRNA expression levels, data dispersion in the Cartesian axis, linear regression curves with $r^2$ values, *P* values, and 95% confidence bands. Correlation analyses of mRNA expression levels for interleukin 10 (IL-10) and transforming growth factor β (TGF-β; *A*), IL-10 and FOXP3 (*B*), IL-10 and interferon γ (IFN-γ; *C*), IL-10 and interleukin 27 (IL-27; *D*), IL-10 and interleukin 21 (IL-21; *E*), TGF-β and FOXP3 (*F*), TGF-β and IFN-γ (*G*), TGF-β and IL-27 (*H*), TGF-β and IL-21 (*I*), FOXP3 and IFN-γ (*J*), FOXP3 and IL-27 (*K*), FOXP3 and IL-21 (*L*), IFN-γ and IL-27 (*M*), IFN-γ and IL-21 (*N*), and IL-27 and IL-21 (*O*). **P* < .05, by the Spearman correlation test.

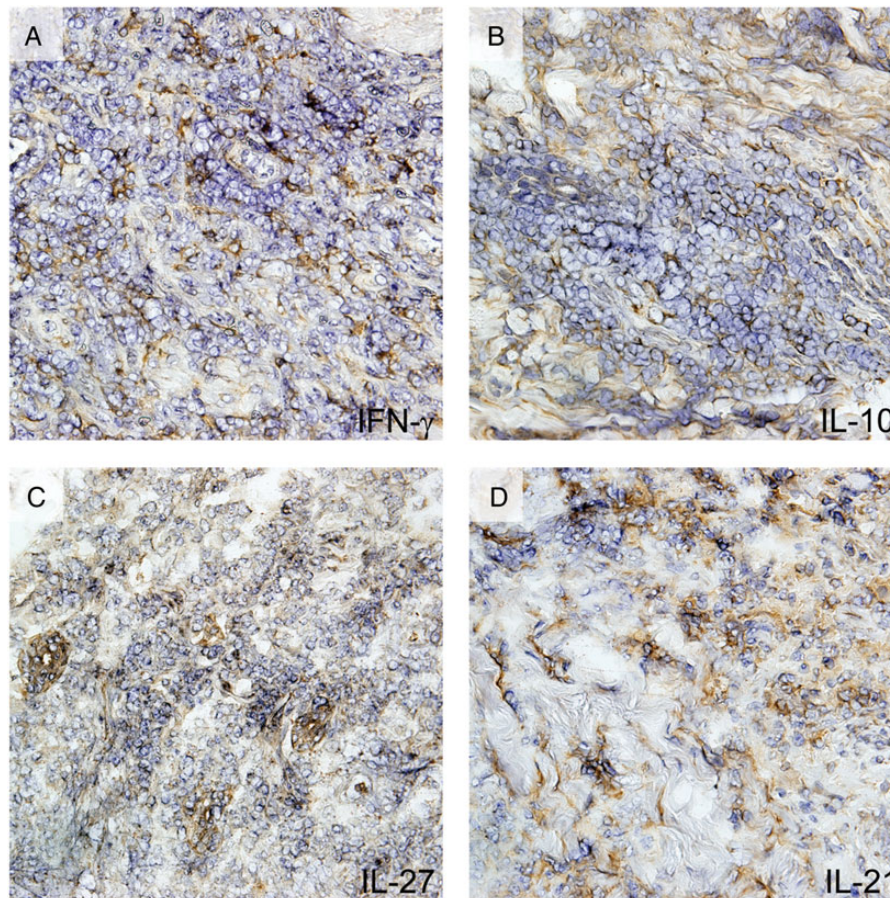


**Figure 4.** Cytokine production in the lesions of cutaneous leishmaniasis patients. Four-millimeter punch biopsy specimens from the lesion borders were frozen in optimal-cutting-temperature compound. Sections from biopsy samples were fixed with cold acetone, and expression of interferon γ (IFN-γ; *A*), interleukin 10 (IL-10; *B*), interleukin 27 (IL-27; *C*), and interleukin 21 (IL-21; *D*) were evaluated by immunohistochemistry, using the peroxidase-diaminobenzidine method. Slides were counterstained with hematoxylin for nucleus visualization. Panels show representative results from 10 patients with CL. Original magnification ×40.

by PBMC from either patients with CL or uninfected controls. It is possible that the Tr-1–like cells that we found in patients with CL exert their suppressive activity via IL-10 production, because Tr-1 suppressor mechanisms have been found to rely mainly on IL-10 production [38]. Additionally, the PBMC of patients with CL respond to exogenous IL-10 by decreasing TNF-α production [39], and IL-10 blockade in visceral leishmaniasis patients results in increased TNF-α production by cells in the spleen [40]. These findings corroborate our results showing decreased production of TNF-α by PBMC in the presence of $CD4^{+}CD25^{-}CD127^{-/low}$ cells from patients with CL.

We have also shown that $CD4^{+}CD25^{-}$ cells are the major sources of IL-10 in the lesions of patients with CL, although these cells were not characterized as extensively in the lesions as they were within the PBMC. Our results also demonstrated IL-21 and IL-27 production in the lesions of patients with CL. Although the expression of IL-27 has been reported in the PBMC from patients with CL [41], this is the first report of IL-21 and IL-27 production in CL lesions. Interestingly, we observed that in lesions of patients with CL, IL-10 expression is associated with the expression of IL-27, IL-21, and IFN-γ but not with expression of FOXP3. These results provide support for the hypothesis that the IL-10–producing $CD4^{+}CD25^{-}$ cells found in the lesions are Tr-1 cells, because the production of IL-27 is known to induce the differentiation of Tr-1 cells and the production of IL-10 by this cell population [27, 42]. IL-21 is also induced by IL-27 and is involved in the production of IL-10 by Tr-1 cells, which may also produce IFN-γ [28, 38, 43]. In accordance with this finding, it was recently shown in human visceral leishmaniasis due to *Leishmania donovani* infection that the production of IL-21 and IL-27 is associated with IL-10 production by $CD4^{+}CD25^{-}FOXP3^{-}$ cells, which may also produce IFN-γ [44]. Therefore, we believe that *L. braziliensis* infection triggers IL-27 production by APCs and that IL-27 subsequently promotes the differentiation of Tr-1 cells that produce IL-10 and IL-21.

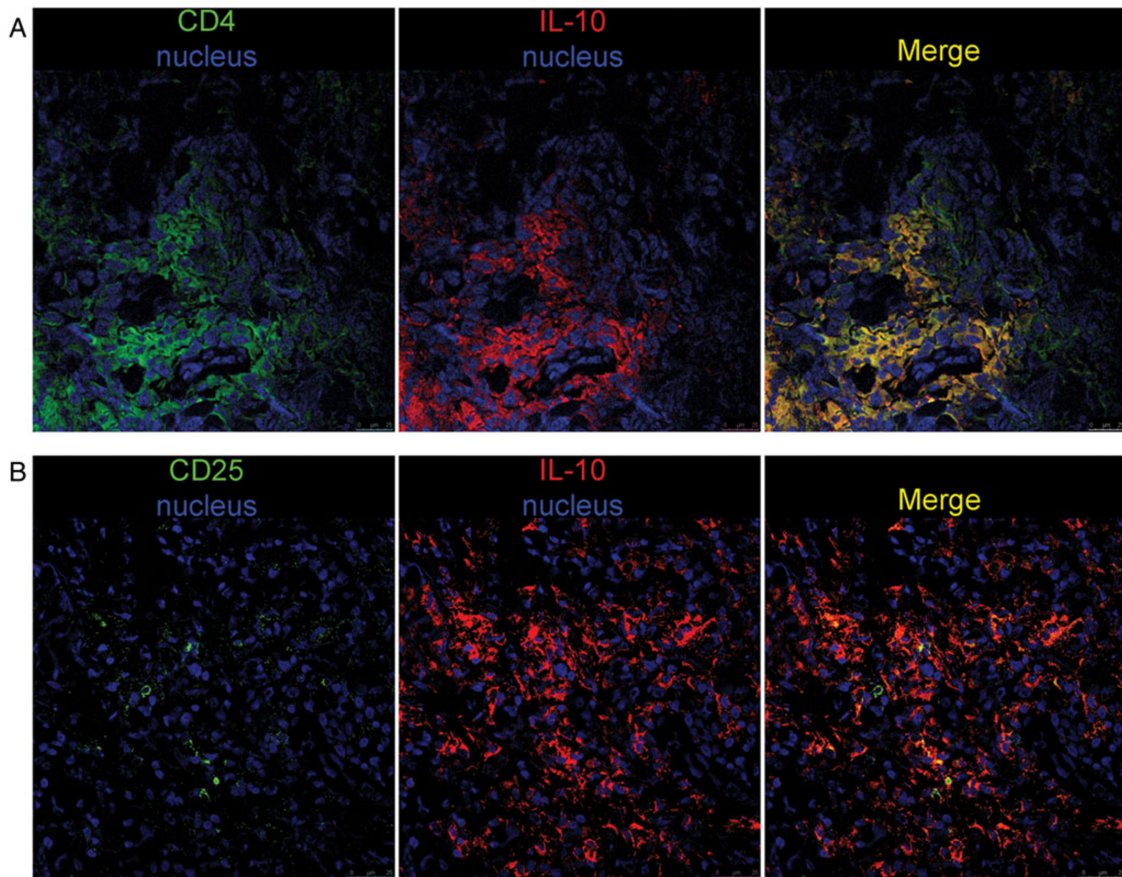


**Figure 5.** Expression of CD4, CD25 and interleukin 10 (IL-10) in the lesions of patients with cutaneous leishmaniasis. Four-millimeter punch biopsy specimens from the lesion borders were frozen in optimal-cutting-temperature compound. Sections from biopsy samples were fixed with cold acetone, and the expressions of CD4, CD25, and IL-10 were evaluated by staining with fluorochrome-labeled antibodies. Nuclei were stained with DAPI, and images were captured in a confocal microscope. *A*, Section stained for CD4 in green (left column) and IL-10 in red (middle column); merged image shows coexpression of CD4 and IL-10 in yellow (right column). *B*, Section stained for CD25 in green (left column) and IL-10 in red (middle column); merged image shows coexpression of CD25 and IL-10 in yellow (right column). In all columns, nuclear staining is shown in blue. Panels show representative results from 4 patients with CL. Original magnification ×40.

The presence of Tr-1 or Tr-1-like cells has also been demonstrated in autoimmune diseases [45]. In experimental autoimmune encephalitis, for example, the activity of IL-10–producing $CD4^+CD25^-FoxP3^-$ cells has been associated with protection against central nervous system immunopathology [46]. Therefore, when immune responses are strong enough to damage vital organs, the organism may benefit from the differentiation of Tr-1 or Tr-1–like cells. However, in infectious diseases in which the generation of strong responses are crucial for containing the infection and host protection, the differentiation of Tr-1–like cells is detrimental for the host, as shown for *L. major* [20, 29] and *L. donovani* infections [47], during which these cells effectively suppress Th1 immune responses and allow parasite replication. In *L. braziliensis* infection, inducing the polarization of Tr-1–like cells may provide the parasites a more efficient escape mechanism from the immune response than simply recruiting natural $CD4^+CD25^+FOXP3^+$ Tregs. That is because Tr-1 cells derive from naive $CD4^+$ T cells that recognized an antigen presented by an APC and were then polarized into Tr-1 cells in the periphery [38]. In the case of *L. braziliensis* infection, this will result in $CD4^+$ T cells with T-cell receptors that are specific for *L. braziliensis* antigens, which will produce IL-10 upon recognition of *L. braziliensis* antigens presented by infected APC, suppressing the activity of the infected cell and of the surrounding lymphocytes. In contrast, natural $CD4^+CD25^+FoxP3^+$ Tregs are polarized in the thymus. Their T-cell receptors are specific for self-antigens, and they respond more efficiently when recognizing self-antigens presented by APCs [48]; therefore, they would not be activated by infected APCs as Tr-1 cells would.

Because CL due to *L. braziliensis* is characterized by a strong Th1 immune response, one could argue that IL-10–producing $CD4^+CD25^-$ $FOXP3^-$ cells may not play an important role in the pathogenesis of the disease in vivo. However, in the initial

phase of the infection, CL is characterized by low production of IFN-γ, and neutralization of IL-10 enhances IFN-γ production [49]. Although the effect of IL-10 neutralization on TNF-α production was not documented in this study, it is very likely that the same feature may occur, since this cytokine is strongly produced in synergy with IFN-γ in human CL [50]. Therefore, the production of IL-10 by $CD4^{+}$ $CD25^{-}$ $FOXP3^{-}$ cells in the initial phase may prevent macrophages from killing the parasites, which favors the establishment of successful infection. However, in later and chronic phases, whereas mentioned earlier, strong Th1 responses take place; the activity of Tr-1 cells may also have a role in protecting tissues from damage caused by inflammatory reaction.

Therefore, the finding that *L. braziliensis* infection induces the differentiation of a Tr-1–like cell population is of the utmost importance for the understanding of CL pathogenesis. Moreover, IL-10 may constitute a target for faster and more-effective treatments for leishmaniasis.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

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