MAJOR ARTICLE

$CD4^+CD25^+$ T Cells in Skin Lesions of Patients with Cutaneous Leishmaniasis Exhibit Phenotypic and Functional Characteristics of Natural Regulatory T Cells

Ana P. Campanelli,[1] Ana M. Roselino,[2] Karen A. Cavassani,[1] Marcelo S. F. Pereira,[1] Renato A. Mortara,[3] Claudia I. Brodskyn,[4,5] Heitor S. Gonçalves,[6] Yasmine Belkaid,[7] Manoel Barral-Netto,[4,5] Aldina Barral,[4,5] and João S. Silva[1]

[1]Department of Biochemistry and Immunology and [2]Division of Dermatology, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, and [3]Department of Microbiology, Immunology, and Parasitology, Universidade Federal de São Paulo, São Paulo, and [4]Gonçalo Moniz Research Institute and [5]Institute of Investigation in Immunology, Salvador, Bahia, and [6]Center for Dermatology Dona Libânia, Fortaleza, Ceará, Brazil; [7]Mucosal Immunology Unit, Laboratory of Parasitic Diseases, National Institutes of Health, Bethesda, Maryland

Endogenous regulatory T (T_{reg}) cells are involved in the control of infections, including *Leishmania* infection in mice. *Leishmania viannia braziliensis* is the main etiologic agent of cutaneous leishmaniasis (CL) in Brazil, and it is also responsible for the more severe mucocutaneous form. Here, we investigated the possible involvement of T_{reg} cells in the control of the immune response in human skin lesions caused by *L. viannia braziliensis* infection. We show that functional T_{reg} cells can be found in skin lesions of patients with CL. These cells express phenotypic markers of T_{reg} cells—such as CD25, cytotoxic T lymphocyte–associated antigen 4, Foxp3, and glucocorticoid-induced tumor necrosis factor receptor—and are able to produce large amounts of interleukin-10 and transforming growth factor–β. Furthermore, $CD4^+CD25^+$ T cells derived from the skin lesions of 4 of 6 patients with CL significantly suppressed in vitro the phytohemagglutinin-induced proliferative T cell responses of allogeneic peripheral-blood mononuclear cells (PBMCs) from healthy control subjects at a ratio of 1 T_{reg} cell to 10 allogeneic PBMCs. These findings suggest that functional T_{reg} cells accumulate at sites of *Leishmania* infection in humans and possibly contribute to the local control of effector T cell functions.

Leishmaniasis, which is caused by infection with protozoan parasites of the genus *Leishmania*, is a severe health problem in many regions of the world. *Leishmania* infection causes a spectrum of clinical diseases, including self-healing skin lesions, diffuse cutaneous disease, mucocutaneous disease, and potentially fatal visceral disease [1]. Clinical symptoms result from replication of the parasites in dermal macrophages, nasopharyngeal mucosa, and cells of the mononuclear phagocytic system [1, 2]. Active disease is associated with an ineffective parasite-specific cell-mediated immune response [3, 4], in that, although $CD4^+$ T cells are essential for resistance to the invading microorganism, they can also contribute to the pathogenesis of leishmaniasis [3]. $CD4^+$ T cells are the principal sources of interferon (IFN)–γ in patients with cutaneous leishmaniasis (CL) [5], and the control of disease in rodents and humans is associated with a Th1-type immune response [6–8]. The release of IFN-γ by infiltrating effector T cells can activate macrophages, leading to the killing of intracellular amastigotes [9]. During CL, the effector T cell response coincides with the development of a localized necrotic ulcer. After successful immu-

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Reprints or correspondence: Dr. João S. Silva, Dept. of Biochemistry and Immunology, School of Medicine of Ribeirão Preto, University of São Paulo, Av. Bandeirantes, 3900, 14049-900 Ribeirão Preto, São Paulo, Brazil (jsdsilva@fmrp.usp.br).

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nologic (or chemotherapeutic) control of *Leishmania* infection, a small number of parasites persist [8], a situation that is associated with immunity to reinfection (known as "concomitant immunity") [10, 11].

Regulatory T (T_{reg}) cells play an important role in the regulation of immune responses and are also responsible for immunologic tolerance. $CD4^+CD25^+$ T_{reg} cells are found in the thymus and peripheral blood (PB) of both humans and mice [12–14]. Controversy exists with respect to the mechanism of action of T_{reg} cells, although several findings have suggested the involvement of cytotoxic T lymphocyte–associated antigen 4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor (GITR), membrane-bound or secreted transforming growth factor (TGF)–β1 [12–15], and interleukin (IL)–10 [16]. Recently, it has been shown that T_{reg} cells can control a large number of infections by modulating the intensity of the effector immune response [17], which also prevents the expression of immune-mediated lesions. In a murine model of *Leishmania major* infection, natural T_{reg} cells favor parasite survival and expansion in a genetically resistant strain; these cells accumulate at sites of infection and locally control the expression of effector T cell functions. In susceptible mice, T_{reg} cells prevent the early expression of lesions but bring about better control of the parasite in the long term [18].

To date, most studies of the role played by T_{reg} cells during infection in humans have focused on cells that were purified from PB. In the present study, we investigated the presence of T_{reg} cells at the site of chronic infection with *Leishmania viannia braziliensis* in humans. Our results indicate that $CD4^+CD25^+$ T cells can be found in skin lesions of humans. Furthermore, the $CD4^+CD25^+$ T cells that accumulated in the skin lesions of the patients infected with *L. viannia braziliensis* exhibited phenotypic and functional characteristics of natural T_{reg} cells, suggesting that they contribute to the local control of effector T cell functions.

PATIENTS, MATERIALS, AND METHODS

Patients with CL and healthy control subjects. We used biopsy samples and PB mononuclear cells (PBMCs) from healthy control subjects and from patients with CL. The control group consisted of 10 healthy subjects (7 men and 3 women; age

Table 1. Clinical characterization of patients with cutaneous leishmaniasis (CL).

Patient (sex, age in years)	Time from appearance of lesion(s), months	Clinical form	MST	IFA	*Leishmania* infection by HE	PCR/RFLP analysis
P1 (M, 40)	4	CL	+	+	+	ND
P2 (F, 58)	60	CL	+	+	+	ND
P3 (M, 17)	90	CL	+	+	+	ND
P4 (M, 16)	180	ML	+	+	+	ND
P5 (F, 19)	15	CL	+	+	+	ND
P6 (M, 16)	6	CL	+	+	+	ND
P7 (M, 17)	15	CL	+	+	+	ND
P8 (M, 12)	15	ML	+	+	+	ND
P9 (M, 35)	240	CL	+	ND	−	*L. viannia braziliensis*
P10 (M, 27)	31	CL	+	ND	−	−
P11 (F, 39)	12	CL	+	−	−	*L. viannia braziliensis*
P12 (M, 65)	36	ML	−	+	+	*L. viannia braziliensis*
P13 (F, 35)	25	CL	+	ND	+	ND
P14 (M, 29)	9	CL	+	+	+	*L. viannia braziliensis*
P15 (M, 64)	3	CL	+	+	+	*L. viannia braziliensis*
P16 (F, 40)	8	CL	+	ND	+	ND
P17 (M, 38)	30	CL	+	ND	+	ND
P18 (M, 20)	3	CL	+	−	−	*L. viannia braziliensis*
P19 (M, 33)	4	CL	+	+	−	*L. viannia braziliensis*
P20 (F, 47)	36	CL	+	+	+	ND

NOTE. −, negative; +, positive; F, female; HE, histopathologic examination; IFA, indirect immunofluorescence assay; M, male; ML, mucocutaneous leishmaniasis; MST, Montenegro skin test; ND, not determined; PCR, polymerase chain reaction; RFLP, restriction fragment–length polymorphism.

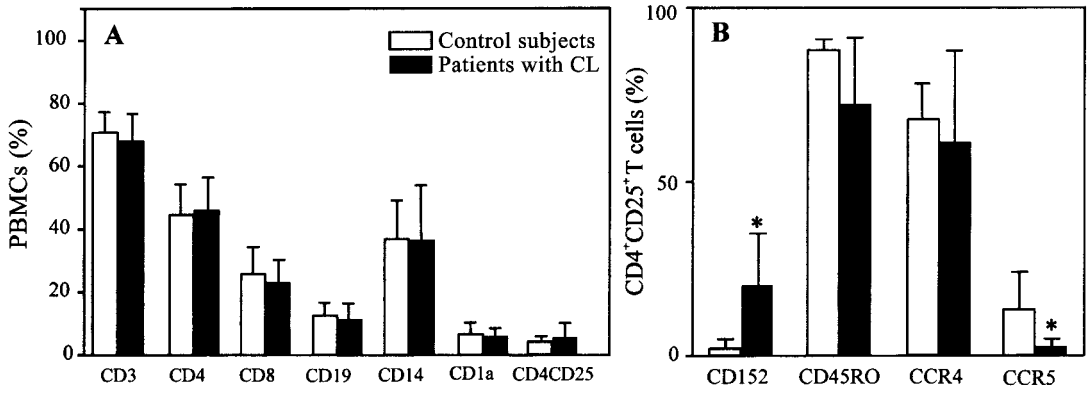

Figure 1. Phenotypic characterization of peripheral-blood mononuclear cells (PBMCs) from patients with cutaneous leishmaniasis (CL). Panel A shows expression of CD3, CD4, CD8, CD19, CD14, CD1a, and both CD4 and CD25 on freshly isolated PBMCs from patients with CL and from healthy control subjects, as determined by flow cytometry. Data are the mean ± SEM results for 12 patients with CL and 7 healthy control subjects tested individually. Panel B shows the proportion of $CD4^+CD25^+$ T cells that coexpressed CD152, CD45RO, CCR4, and CCR5. Data are the mean ± SEM results for 12 patients with CL and 7 healthy control subjects tested individually. $^*P < .05$, compared with the healthy control subjects.

range, 19–50 years) with a negative result for the Montenegro skin test. The group of patients consisted of 20 individuals with active CL (14 men and 6 women; age range, 20–65 years), all of whom exhibited clinical and laboratory features of the disease. The patients were from areas where only *L. viannia braziliensis* is prevalent and had differing durations of disease, which varied from 3 months to 20 years. Clinical diagnosis of CL was confirmed by fulfillment of 1 or more of the following criteria: a positive result for the Montenegro skin test or an indirect immunofluorescence assay, presence of *Leishmania* species on histopathologic examination, isolation of *Leishmania* species in culture, or a positive polymerase chain reaction (PCR) result for *Leishmania* species (table 1). PCR/restriction fragment–length polymorphism analysis confirmed the presence of *L. viannia braziliensis* in 7 patients, and 13 had positive cultures for *Leishmania* species. The Institutional Review Board of the School of Medicine of Ribeirão Preto approved the protocol of the present study, and written, informed consent was obtained from all of the patients.

Antibodies. For immunostaining, peridinin chlorophyll protein (PercP)–, phycoerythrin (PE)–, and fluorescein isothiocyanate (FITC)–conjugated antibodies (BD PharMingen) against CD3 (UCHT1), CD4 (RPAT4), CD8 (RPA-T8), CD14 (M5E2), CD1a (HI149), CD19 (HIB19), CD25 (M-A251), CD28 (CD28.2), CD45RO (UCHL1), CD62L (DREG-56), CD152 (BNI3.1), HLA-DR (G46–6), CCR4 (1G1), and CCR5 (2D7/CCR5) were used, as were the respective mouse and rat isotype controls. Monoclonal antibody (MAb) against human GITR/TNFRSF18 (110416) was purchased from R&D Systems. The antibodies used for intracellular cytokine staining were FITC- and PE-conjugated anti–IL-10 (JES3–19F1) and anti–TGF-β (BD PharMingen). Unconjugated anti-CD3 (UCHT1), anti-CD28 (CD28.2), and recombinant IL-2 (BD PharMingen) were used for polyclonal activation of T cells. Antibodies against human Foxp3, TGF-β, IL-10, CCL3, CCL4, CCL5, CD25, CCL17, CCL22, CCR4, CD4, CD8, CD1a, CD3, and CD68 (Santa Cruz Biotechnology) were used to detect inflammatory cells in biopsy samples.

Isolation of leukocytes from skin lesions. To characterize leukocytes at the lesion site, the biopsy samples from skin lesions were collected and incubated for 1 h at 37°C in RPMI 1640 containing $NaHCO_3$, penicillin, streptomycin, gentamicin, and 0.28 Wunsch units/mL liberase blendzyme CI (Roche Diagnostics). Biopsy samples were processed in the presence of 0.05% DNase (Sigma-Aldrich) by use of a Medimachine (BD PharMingen), in accordance with the manufacturer's instructions. After processing, cell viability was assessed by trypan blue exclusion, and cells were filtered through a 50-μm filter and washed before activation and/or immunolabeling.

Purification of T cell subpopulations. $CD4^+$ T cells isolated from skin lesions were purified using CD4 MACS MultiSort beads (Miltenyi Biotec), in accordance with the manufacturer's instructions. $CD4^+$ T cells, after being detached, were washed in 0.5% PBS/human serum albumin plus 3 mmol/L EDTA and incubated with anti-CD25 beads (3 μL/1 $\times$ 10^7 cells; Miltenyi Biotec). $CD4^+CD25^+$ T cells, after being detached, were washed and used immediately. Purity was assessed by flow cytometry.

Flow cytometry. For immunofluorescence staining, cells were washed and stained for 20 min at 4°C with the optimal dilution of each antibody. Cells were washed and then analyzed by flow cytometry. For detection of intracellular cytokines, cells were stained for CD25 expression, fixed, and permeabilized with saponin (fix/perm solution; BD PharMingen) before being stained with specific antibodies.

In vitro expansion of T cell lines. Fractionated $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells (1 $\times$ 10^5 cells/well) were first activated using anti-CD3 MAb (2 μg/mL; OKT-3) in the presence of

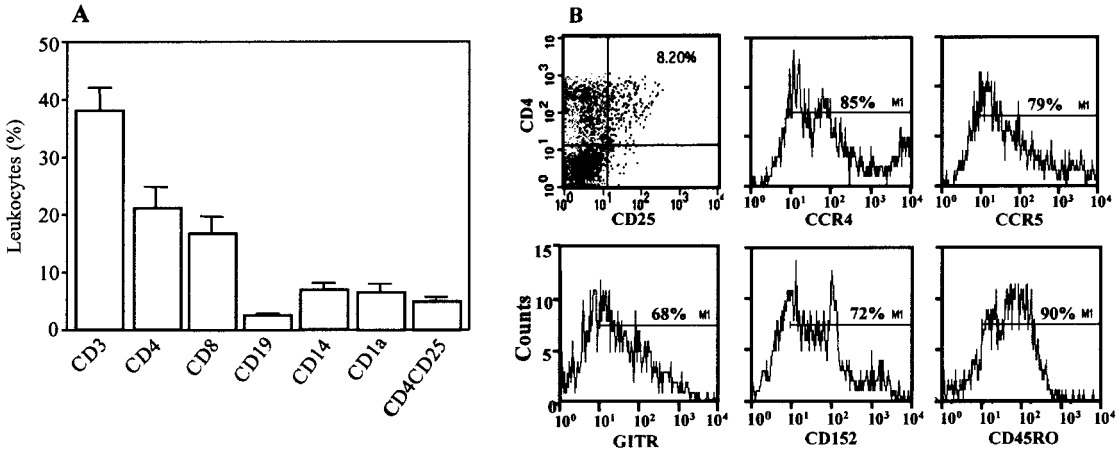

Figure 2. Phenotypic characterization of leukocytes derived from skin lesions of patients with cutaneous leishmaniasis (CL). Biopsy samples of skin lesions were digested with liberase, disrupted, filtered, and washed, and cell concentration and viability were determined. Panel A shows the proportion of skin lesion–derived leukocytes that expressed CD3, CD4, CD8, CD19, CD14, CD1a, and both CD4 and CD25. Data are the mean ± SEM results for 12 patients with CL tested individually. The histograms in panel B show the proportion of CCR4, CCR5, glucocorticoid-induced tumor necrosis factor receptor (GITR), CD152, and CD45RO gated on $CD4^+CD25^+$ T cells from 1 patient with CL. The total proportion of $CD4^+CD25^+$ T cells that were positive for each marker is indicated in each histogram. The experiment is representative of the results for the 12 patients with CL.

4×10^6 irradiated (6000 rad) allogeneic or syngeneic dendritic cells in 96-well U-bottom tissue culture plates. All cultures were performed in RPMI 1640 plus 10% fetal bovine serum (FBS), 2 mmol/L glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin (GIBCO BRL). IL-2 (40 U/mL) was added on days 2, 5, and 7 after primary stimulation. On day 10, $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were harvested, and their suppressor activity was evaluated in proliferation assays.

Cocultures and proliferation assays. To analyze the suppressor activity of skin lesion–derived $CD4^+CD25^+$ T cells, 1×10^4 cells/well were distributed in 96-well plates (Corning) and cocultured with allogeneic PBMCs (1×10^5 cells/well) with or without 1 μg/mL phytohemagglutinin (PHA; GIBCO BRL). After 4 days, 0.5 μCi/well [^{3}H]-thymidine (Amersham Biosciences) was added, and the cells were cultured for an additional 16 h before being harvested; radioactivity was measured in a liquid scintillation beta counter (Beckman Instruments) [19]. Data were expressed as the mean counts per minute from triplicate cultures, and the proliferation index was calculated as the mean value for cell proliferation in the presence of $CD4^+CD25^+$ T cells divided by the mean value for cell proliferation in the absence of $CD4^+CD25^+$ T cells.

Immunohistochemistry. Fresh frozen skin sections were obtained from the biopsy samples from 10 patients with CL. Fragments of the skin samples were covered with optimum cutting temperature medium (Sakura Finetek), snap-frozen in liquid nitrogen, and stored at −196°C until analysis. Five-micron cryostat sections were dried on glass slides covered with poly-L-lysine (Sigma) and fixed in cold acetone. Avidin-biotin peroxidase techniques were used to reveal chemokine-producing cells present in the sections.

Confocal microscopy. Slides for double immunofluorescence staining were postfixed with 4% paraformaldehyde and blocked with 15% FBS/PBS. After being washed, slides were incubated with the primary antibody, washed again, and incubated with the appropriate fluorochrome-conjugated (Texas Red, Cy3, or FITC) secondary antibodies as well as 4′,6′-diamidino-2-phenylindole. After being washed, the slides were mounted using 90% glycerol/PBS and analyzed using a confocal microscope. Images were captured via a Bio-Rad 1024UV confocal system attached to a Zeiss Axiovert 100 microscope equipped with a ×63 oil-immersion planapochromatic objective (1.4 NA) with differential interference contrast. LaserSharp 1024 (version 3.2T; BioRad) was used for image acquisition, and Adobe Photoshop (version 4.0) was used for image processing. Secondary antibodies alone were used as negative controls.

Statistical analysis. Statistical analysis was performed using analysis of variance, followed by the Tukey-Kramer multiple comparison test (InStat; GraphPad). $P < .05$ was considered to indicate significance.

RESULTS

Phenotypic characterization of PBMCs from patients with CL. First, we determined the frequencies of expression of various leukocyte markers on the surface of freshly isolated PBMCs from selected healthy control subjects ($n = 7$) and patients with CL ($n = 12$). We found that expression of CD3, CD4, CD8, CD19, CD14, and CD1a was similar for both groups. In particular, the proportions of putative $CD4^+CD25^+$ T cells for the healthy control subjects (range, 2.2%–7.0% of cells) and the patients with CL (range, 2.2%–13.3%) were similar (figure 1*A*).

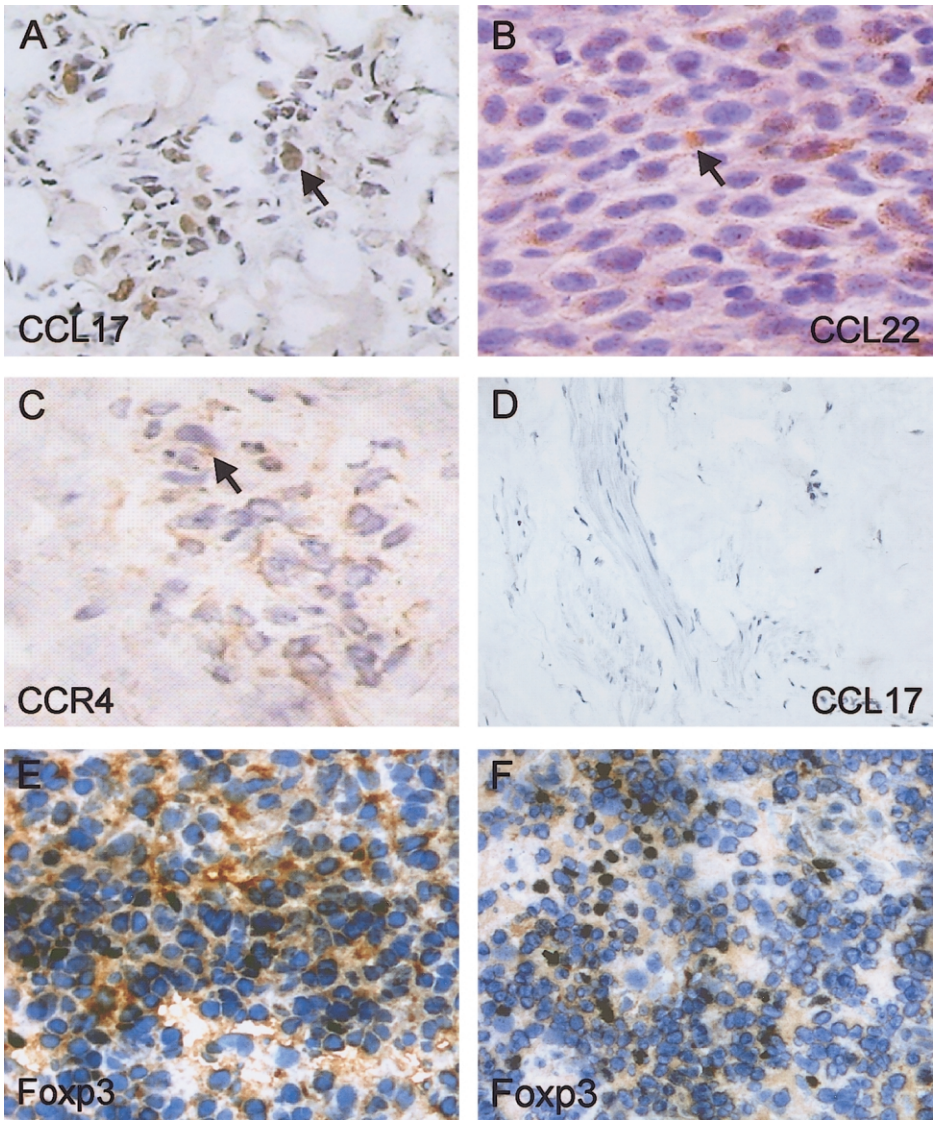

Figure 3. Expression of CCL17, CCL22, CCR4, and Foxp3 in skin lesions of patients with cutaneous leishmaniasis (CL). Skin sections from biopsy samples were fixed with cold acetone, and expression of CCL17, CCL22, CCR4, and Foxp3 was investigated by immunohistochemistry. Slides were stained with mouse anti–human CCL17 *(A* and *D),* anti–human CCL22 *(B),* anti–human CCR4 monoclonal antibody *(C),* and anti–human Foxp3 *(E* and *F),* followed by biotinylated anti–mouse IgG and avidin-biotin peroxidase. The slides were then developed with 3,3-diaminobenzidine *(brown)* and counterstained with hematoxylin. Panel D shows CCL17 expression in a control biopsy sample (obtained from a woman undergoing cosmetic breast surgery); other panels show results that are representative of those for 10 patients with CL. Original magnification, ×400.

In addition, similar proportions of $CD4^+CD25^+$ T cells expressing CD45RO and CCR4 were found for the healthy control subjects and the patients with CL. In contrast, expression of CTLA-4 (CD152) on $CD4^+CD25^+$ T cells was significantly higher ($P < .05$) for the patients with CL (range, 5.6%–40.1%) than for the healthy control subjects (range, 0%–5.6%), and expression of CCR5 on $CD4^+CD25^+$ T cells was higher ($P < .05$) for the healthy control subjects (range, 0.5%–25.0%) than for the patients with CL (range, 0.5%–6.1%) (figure 1*B*). Therefore, although the proportions of $CD4^+CD25^+$ T cells were similar for both groups, the relative expression of CTLA-4 and CCR5 was different.

Phenotypic characterization of leukocytes in skin lesions. Next, we characterized the leukocytes derived from skin lesions of patients with CL ($n = 12$). $CD3^+$ T cells (ranging from 2.0×10^5 to 1.7×10^6 cells/biopsy) represented the main population (range, 11.4%–55.8% of cells), with $CD4^+$ T cells ranging from 5.6×10^4 to 6.9×10^5 cells/biopsy (range, 3.3%–46.2%) and $CD8^+$ T cells ranging from 4.2×10^4 to 7.4×10^5 cells/biopsy (range, 4.7%–46.0%). We also found 1.0×10^4 to 7.3×10^4 $CD1a^+$ cells/biopsy (range, 0.9%–13.0%), 1.7×10^4 to 1.8×10^5 $CD14^+$ cells/biopsy (range, 1.2%–19.0%), 7.6×10^3 to 1.9×10^5 $CD19^+$ cells/biopsy (range, 1.0%–4.3%), and 5.2×10^3 to 1.9×10^5 $CD4^+CD25^+$ cells/biopsy (range, 1.4%–10.3%)

Table 2. Expression of chemokines and Foxp3 in skin lesions of patients with cutaneous leishmaniasis (CL).

	Patients with CL										Control subjects		
Molecule	P1	P2	P3	P4	P5	P6	P7	P8	P17	P18	C1	C2	C3
CCL17	3	2	3	3	2	2	3	3	2	3	1	1	1
CCL22	0	1	0	1	1	1	1	1	1	1	1	1	1
CCR4	1	1	1	1	1	1	1	1	2	2	0	0	0
Foxp3	3	2	1	3	1	2	2	3	3	3	0	0	0

NOTE. Values indicate intensity of immunostaining, as follows: 0, absent; 1, low intensity; 2, medium intensity; and 3, high intensity. Control biopsy samples were obtained from women undergoing cosmetic breast surgery.

(figure 2*A*). Importantly, the majority of cells that coexpressed CD4 and CD25 also expressed CD45RO, CD152, CCR4, GITR, and CCR5 (figure 2*B*). Together, these results show that $CD4^+CD25^+$ T cells are found at the sites of infection and exhibit a phenotype that is characteristic of natural T_{reg} cells.

CCL17, CCL22, CCR4, and Foxp3 expression in skin lesions of patients with CL. Because T_{reg} cells migrate in response to CCL17 and CCL22 [20], we investigated the expression of these chemokines in the skin lesions of patients with CL ($n = 10$). We found CCL17 in the biopsy samples from the patients with CL (figure 3*A* and table 2), although it was also present in control biopsy samples ($n = 3$; obtained from women undergoing cosmetic breast surgery) at a lower intensity (figure 3*D* and table 2). CCL17 was detected in vascular endothelial cells, epithelial cells, and, to a lesser degree, in infiltrated cells. Expression of CCL22, which was localized in epithelial cells, was similar for both groups (figure 3*B* and table 2). CCR4 (figure 3*C* and table 2) and Foxp3 (figure 3*E* and 3*F* and table 2) were also detected in leukocytes from the patients with CL but were not detected in leukocytes from the healthy control subjects. These results indicate that natural T_{reg} cells are present in skin lesions of patients with CL.

IL-10 and TGF-β production by $CD4^+CD25^+$ T cells derived from skin lesions of patients with CL. A previous study demonstrated that T_{reg} cells produce IL-10, a cytokine that is related to the persistence of *L. major* infection [5]. Here, we found that >70% of the $CD4^+CD25^+$ T cells derived from the skin lesions of patients with CL ($n = 12$) produced IL-10 independently of PHA stimulation (figure 4*A*). We also found that 20%–51% of the $CD4^+CD25^+$ T cells derived from the skin lesions of the patients with CL produced TGF-β when cultured for 6 h in the absence of stimulation (figure 4*A*). Culture with PHA increased the percentage of TGF-β–producing cells, which then ranged from 79% to 98%. TGF-β production in skin lesions was confirmed by confocal microscopy. Immunostaining of lesions revealed that $CD25^+$ T cells are an important source of IL-10 and TGF-β (figure 4*B*). These results strongly suggest that IL-10 and TGF-β are produced by $CD4^+CD25^+$ T cells at the site of *L. viannia braziliensis* infection.

Suppression of allogeneic T cell proliferation in vitro by $CD4^+CD25^+$ T cells from skin lesions of patients with CL. To determine whether skin lesion–derived $CD4^+CD25^+$ T cells are functional T_{reg} cells, $CD4^+CD25^+$ T cells were purified from skin lesions; expanded using anti-CD3 MAb, allogeneic feeder cell mixture, and exogenous IL-2; and cultured with allogeneic PBMCs from healthy control subjects plus PHA. T cell proliferation was then evaluated. $CD4^+CD25^+$ T cells obtained from 4 of 6 patients with CL significantly suppressed PHA-induced T cell proliferation at a ratio of 1 T_{reg} cell to 10 allogeneic PBMCs (figure 5). The $CD4^+CD25^+$ T cells isolated from 2 patients with CL were unable to suppress such proliferation at the 1:10 ratio, showing that $CD4^+CD25^+$ T cells from different patients do not exhibit similar suppressive properties.

DISCUSSION

A growing body of evidence has shown that $CD4^+CD25^+$ T cells play a critical role in the immune response, including that induced by transplanted organs, tumors, and parasite antigens [12]. In addition, the number of immune cells and their effector functions are under the strict control of T_{reg} cells [13]. Recent findings, derived from experimental models of infection with *Pneumocystis carinii* [21], *L. major* [18], and *Candida albicans* [22], have emphasized a role for T_{reg} cells in controlling tissue destruction at the sites of infection. Here, we investigated whether T_{reg} cells modulate the effector immune response induced by *L. viannia braziliensis* infection in humans. We concluded that the levels of $CD4^+CD25^+$ T_{reg} cells in the PB of patients with CL were similar to those found in healthy control subjects. These results are in agreement with those of other studies showing that $CD4^+CD25^+$T cells represent 5%–10% of peripheral $CD4^+$ T cells in the blood [12–14]. CD45RO and CCR4, which are normally present on T_{reg} cells [20–23], were also similarly expressed in both groups studied. On the other hand, $CD4^+CD25^+$ T cells from the patients with CL exhibited higher levels of CTLA-4 and lower levels of CCR5 expression than did the those from the healthy control subjects. Because CTLA-4 is highly expressed on T_{reg} cells [13, 14], and because it is supposed that this molecule plays an im-

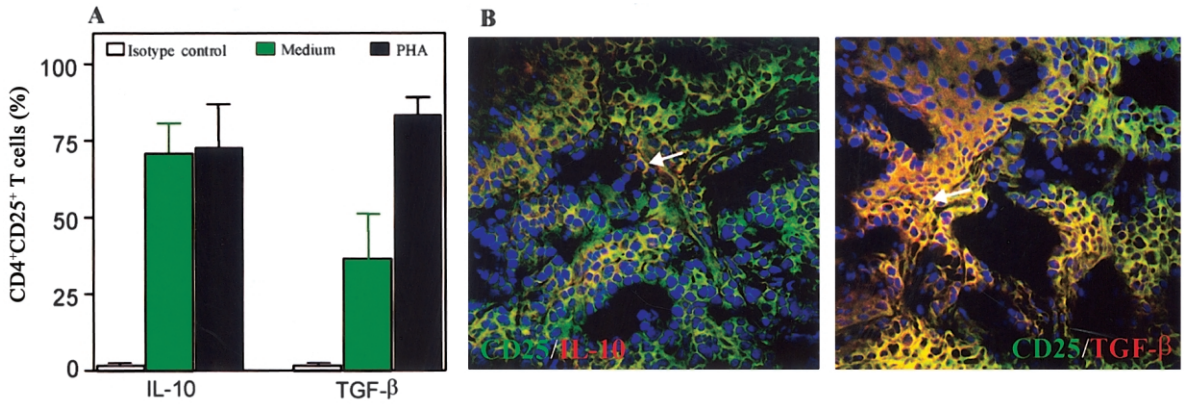

Figure 4. Production of interleukin (IL)–10 and transforming growth factor (TGF)–β by $CD4^+CD25^+$ T cells derived from skin lesions of patients with cutaneous leishmaniasis (CL). The $CD4^+CD25^+$ T cells from the patients with CL were cultured for 6 h, and intracellular cytokine production was assessed by flow cytometry. Panel A shows the proportion of skin lesion–derived $CD4^+CD25^+$ T cells that were positive for IL-10 or TGF-β after culture with medium alone or with phytohemagglutinin (PHA); also shown are results for the respective isotype controls. Data are the mean ± SEM results for 12 patients with CL tested individually. Panel B shows the staining for CD25 *(green)* and either IL-10 or TGF-β *(red)* observed in 1 representative experiment. Biopsy samples were fixed, incubated with anti-CD25 and either anti–IL-10 or anti–TGF-β, stained with the appropriate fluorochrome-conjugated secondary antibodies as well as 4′,6′-diamidino-2-phenylindole *(blue),* and analyzed by confocal microscopy. Arrows indicate regions of coexpression of CD25 and cytokines.

portant role in their suppressor function [14], it is possible that the suppressor activity of $CD4^+CD25^+$ T cells was increased in the patients with CL. The possibility of decreased traffic of these cells to tissues is low, because the level of CCR4 expression was similar in both groups. Moreover, the results showed that the number of $CD4^+CD25^+CCR5^+$ T cells was reduced in the PB of the patients with CL. Because the expression of CCR5 is correlated with the homing of $CD4^+CD25^+$ T cells [24] and is required for their function [25], these results suggest that at least $CD4^+CD25^+CCR5^+$ T cells were not accumulating in the PB.

In this context, we examined the presence of $CD4^+CD25^+$ T cells in skin lesions of the patients with CL. The results showed that a mean of 4.91% of the leukocytes coexpressed the markers CD4 and CD25. Because $CD4^+CD25^+$ T cells that exhibit the suppressor function normally express the markers CCR4 [20], CTLA-4 [23], CCR5 [24], CD45RO [23], and GITR [26], we investigated them in the PB. We found that the majority of $CD4^+CD25^+$ T cells also expressed these markers, with ~90% of them expressing CD45RO. These results suggest that T_{reg} cells were effectively migrating to the lesions caused by *L. viannia braziliensis* infection. Expression of these normal T_{reg} cell markers has also been described during breast adenocarcinoma, [27], rheumatoid arthritis [28], and juvenile idiopathic arthritis [29, 30].

We also observed a high number of $CD8^+$ T cells, macrophages, and dendritic cells in the biopsy samples. These results are in accordance with those of several other studies showing that the number of lymphocytes, as identified by optical immunohistochemistry, is considerably higher than the number of other inflammatory cells in skin lesions [31, 32]. Similarly, in the lesions of mice infected with *L. major,* the number of lymphocytes is higher than the number of other leukocytes [18].

The migration of $CD4^+CD25^+$ T cells to the PB seems to be dependent on CCR4 expression and on CCL17 and/or CCL22 production in the tissues [20]. This means that the production of CCL17 and CCL22 during the course of the inflammatory response could dictate the extent, severity, and duration of the process by modulating recruitment of T_{reg} cells expressing CCR4 and CCR8. Investigation of these chemokines in the skin lesions of the patients with CL revealed that both are highly expressed. Although they can also be produced by normal activated skin keratinocytes, antigen-presenting cells, and activated T cells [33], the quantities of chemokines detected in the lesions were markedly higher than those found in normal tissue (table 2). Moreover, CCR4 was also detected on the inflammatory cells present in the lesions. The presence of CCL3, CCL4, and CCL5 in the lesions (data not shown) correlates with the expression of CCR5 on ~79% of $CD4^+CD25^+$ cells (figure 3). We also were able to identify, by immunostaining, leukocytes expressing Foxp3 in all of the patients with CL. One aspect that must be emphasized is that the expression of Foxp3 is variable, ranging from just a few cells to a high percentage of positive cells per field. In light of previously published observations [34, 35], these results strongly suggest that the $CD4^+CD25^+$ T cells found in the skin lesions of patients with CL are natural T_{reg} cells.

We next examined the production of IL-10 and TGF-β by leukocytes coexpressing CD4 and CD25, because T_{reg} cells are also characterized by their ability to produce these cytokines [12, 35]. We observed that, without PHA stimulation, >70%

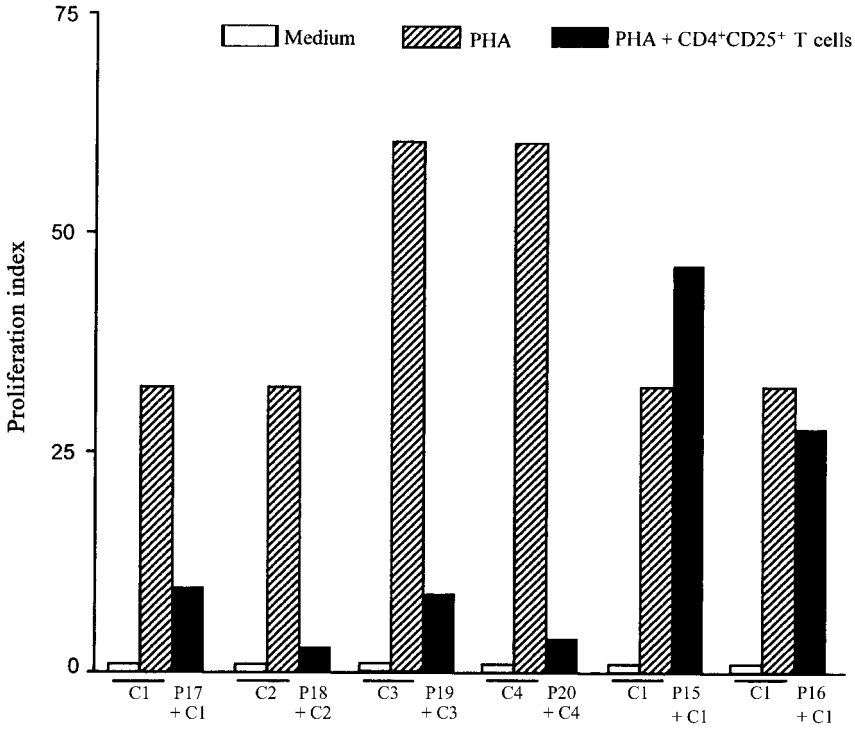

Figure 5. Suppression of allogeneic T cell proliferation in vitro by $CD4^+CD25^+$ T cells derived from skin lesions of patients with cutaneous leishmaniasis (CL). $CD4^+CD25^+$ T cells were cultured with anti-CD3 monoclonal antibody, allogeneic feeder cell mixture, and exogenous interleukin-2. $CD4^+CD25^+$ cells were tested for their ability to suppress the proliferation of allogeneic peripheral-blood mononuclear cells (PBMCs). Allogeneic PBMCs (1×10^5 cells/well) from healthy control subjects (C1, C2, C3, and C4) were cultured with medium only, with phytohemagglutinin (PHA), or with PHA plus 1×10^4 $CD4^+CD25^+$ cells from 6 patients (P15–P20). After 4 days of culture, [^{3}H]-thymidine was added, and the cells were cultured for an additional 16 h before being harvested. The proliferation observed with 1×10^4 $CD4^+CD25^+$ T cells was always <1000 cpms. The proliferation index was calculated as the mean value for cell proliferation in the presence of $CD4^+CD25^+$ T cells divided by the mean value for cell proliferation in the absence of $CD4^+CD25^+$ T cells.

and up to 51% of the $CD4^+CD25^+$ T cell population expressed IL-10 and TGF-β, respectively, reinforcing the notion that $CD4^+CD25^+$ T cells in the skin lesions of patients with CL are, in fact, natural T_{reg} cells. IL-10 is an important immunomodulatory cytokine [36, 37], being essential in limiting the immune responses to several pathogens [38, 39]. IL-10 is correlated with the suppression of the antigen-induced proliferative T cell response [40], has the ability to inhibit antigen presentation [41] and type 1 cytokine production [42], and can render macrophages refractory to IFN-γ activation [43, 44]. Moreover, IL-10 is involved in *Leishmania* persistence and reactivation of disease in humans [45, 46]. Similarly, IL-10 and TGF-β have been implicated in the suppressor mechanism mediated by $CD4^+CD25^+$ T cells in autoimmune diseases [12, 13]. TGF-β also plays an important regulatory role in CL, enhancing parasite virulence and replication in macrophages [47, 48]. Therefore, it is possible that $CD4^+CD25^+IL\text{-}10^+TGF\text{-}\beta^+$ T cells are involved in modulating the effector immune response in the skin lesions induced by *Leishmania* infection in humans. This hypothesis is in accordance with the findings of a study that demonstrated that T_{reg} cells are involved in the control of *L. major* infection by IL-10–dependent and –independent mechanisms [18].

To confirm that the $CD4^+CD25^+$ T cells derived from the skin lesions of patients with CL had regulatory properties, we cultured the cells with allogeneic PBMCs from healthy control subjects at a ratio of 1 T_{reg} cell to 10 allogeneic PBMCs. The skin lesion–derived $CD4^+CD25^+$ T cells from 4 of 6 patients suppressed the proliferation of allogeneic $CD4^+$ T cells, indicating that they were the kind of natural T_{reg} cells that have been previously shown to be involved in the suppression of the immune response and in the persistence of parasites in skin lesions [18]. It is intriguing that the $CD4^+CD25^+$ T cells from 2 of 6 patients did not exhibit suppressor activity at the 1:10 ratio. It is possible that the $CD4^+CD25^+$ T cells found in the skin lesions of these 2 patients were related to the effector T cell function, although we did not analyze the expression of CD69, a marker correlated with T cell activation [49]. Alternatively, it is possible that the number of $CD4^+CD25^+$ T cells in the skin lesions, or the levels of cytokines secreted by them, was unable to suppress the allogeneic immune response at the 1:10 ratio. In fact, the frequency of $Foxp3^+$ cells in the lesions

was variable. However, it was not possible to confirm the number of Foxp3$^+$cells in all of the samples studied, because the size of the biopsy sample was frequently too small. Fewer T_{reg} cells in skin lesions could be explained by a decreased level of the chemokines responsible for driving T_{reg} cell migration to the skin, such as CCL17 and CCL22 [20]. Although we need to perform experiments to confirm this hypothesis and to follow the patients for a longer period of time, we did find differences in chemokine production in the skin lesions of the patients with CL (table 2). Similar results have been observed in patients with atopic allergies, in whom CD4$^+$CD25$^+$ T cells, the producers of IL-10, did not inhibit the proliferative T cell response [50]. One aspect that we could not confirm is whether the T_{reg} cells found in skin lesions are involved in the control of parasite persistence and in the pathogenesis of these lesions.

Taken together, the data presented here show that CD4$^+$CD25$^+$ T cells were present in the skin lesions of patients infected with *L. viannia braziliensis* and that such cells from the majority of patients analyzed exhibited the phenotypic and functional characteristics of natural T_{reg} cells. Further studies are needed to unravel the relationship between T_{reg} cells and different clinical presentations of leishmaniasis—understanding this relationship may bring about new therapeutic and prophylactic strategies against the parasites involved.

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