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Systemic and Local Characterization of Regulatory T Cells in a Chronic Fungal Infection in Humans

Karen A. Cavassani,* Ana P. Campanelli,* Ana P. Moreira,* Jaqueline O. Vancim,* Lucia H. Vitali,† Rui C. Mamede,‡ Roberto Martinez,† and João S. Silva2*°

The long-term persistence of pathogens in a host is a hallmark of certain infectious diseases, including schistosomiasis, leishmaniasis, and paracoccidioidomycosis (PCM). Natural regulatory T (Treg) cells are involved in control of the immune responses, including response to pathogens. Because CTLA-4 is constitutively expressed in Treg cells and it acts as a negative regulator of T cell activation in patients with PCM, here we investigated the involvement of Treg cells in the control of systemic and local immune response in patients with PCM. We found that the leukocyte subsets were similar in patients and controls, except for CD11c+CD1a+ cells. However, a higher frequency of CD4+CD25+ T cells expressing CTLA-4, glucocorticoid-inducible TNFR, membrane-bound TGF-β, and forkhead-box 3 were observed in PBMC of patients. In accordance, these cells exhibited stronger suppressive activity when compared with those from controls (94.0 vs 67.5% of inhibition of allogeneic T cell proliferation). In addition, the data showed that CD4+CD25+ T cells expressing CTLA-4+, glucocorticoid-inducible TNFR positive, CD103+, CD45RO+, membrane-bound TGF-β, forkhead-box 3 positive, and the chemokines receptors CCR4 and CCR5 accumulate in the Paracoccidioides brasiliensis-induced lesions. Indeed, the secreted CCL17 and CCL22, both associated with the migration of Treg cells to peripheral tissues, were also detected in the biopsies. Moreover, the CD4+CD25+ T cell derived from lesions, most of them TGF-β+, also exhibited functional activity in vitro. Altogether, these data provide the first evidence that Treg cells play a role in controlling local and systemic immune response in patients with a fungal-induced granulomatous disease advancing our understanding about the immune regulation in human chronic diseases. The Journal of Immunology, 2006, 177: 5811–5818.

One of the most important endemic diseases in Latin America is paracoccidioidomycosis (PCM), a deep granulomatous mycosis caused by the dimorphic fungus Paracoccidioides brasiliensis (1). The infection, acquired by inhalation of conidia produced by the mycelial phase of the fungus, affects the lungs and can spread to multiple organs via the lymphatic system and bloodstream. The disease presents a broad spectrum of clinical and pathological manifestations, ranging from localized lesions to severely disseminated infection (2). In the endemic areas, surveys with the paracoccidioidin skin test show that many individuals that recognize the fungus Ags are only infected, without any manifestation of disease (2). These chronic infected individuals have persistent Ag stimulation in vivo and develop an apparent effective control of fungal growth and dissemination. Other patients develop the acute form of the disease. The acute form (or juvenile) of this mycosis is very severe and mimics a systemic lymphoproliferative disease (2, 3). The chronic form (or adult) is the most frequent clinical presentation of the disease, which is commonly due to reactivation of infection from quiescent foci (4).

The protective host immunity includes Th1-type response, in which IFN-γ and TNF-α (3, 5, 6) are fundamentals to control of fungus dissemination and death. However, the patients with PCM usually show impaired cellular immune response to fungal Ags (3, 7, 8). Imbalance in cytokine production (9), with decreased IFN-γ (10) and increased TGF-β (8, 11) and IL-10 production (9, 12, 13), high levels of Fas-L and CTLA-4 expression in T cells (8), and apoptosis-induced cell death (8, 14) are postulated to be involved in the T cell unresponsiveness seen in these patients. Interestingly, impaired T cell proliferation, CTLA-4 expression, and TGF-β and IL-10 production are also involved in regulation of the effector immune response mediated by regulatory T (Treg) cells (15, 16).

Natural Treg cells are emerging as a regulator key of immune responses. They are CD4+CD25+ T cells that potentially regulate the activation, proliferation, and effector function of activated conventional T cells in several immunological settings such as immunopathology, autoimmune diseases, transplantation, tumor immunity, and infectious disease (15–18). Besides CD25 and CTLA-4, Treg cells also can express several other phenotypic molecules, including glucocorticoid-inducible TNFR (GITR), CD103, CD45RO (15, 16, 19), cell surface TGF-β1 (15, 20), and forkhead-box 3 (Foxp3) (21). The functional mechanism of Treg cells is not clear, but the ligation of CTLA-4, GITR, and involvement of anti-inflammatory cytokines such as IL-10 and TGF-β has been suggested (15, 16, 22). Currently, it is known that natural Treg cells can respond to a variety of self Ags as well as those expressed by...
microbes. In experimental models, such as infection by *Leishmania*, HSV and *Schistosoma mansoni*, Treg cells seem to play a role in the maintenance of chronic infections, with persistence of pathogens, consequently enabling the disease reactivation (18). Indeed, in a mouse model of chronic *Leishmania* infection, the transfer of purified Treg cells derived from infected mice into other chronically infected animals was sufficient to trigger disease reactivation and inhibit the effector memory response (18, 23).

The effector immune response against *P. brasiliensis* has to be regulated to control the fungal growth and dissemination and to prevent the damage tissue. In this study, we explore the presence and characteristics of CD4+CD25+ T cells in the peripheral blood and site of lesion from patients with PCM. The results showed that CD4+CD25+ T cells with the classical phenotype and function identical with the Treg cells are present in the lesions and peripheral blood. Moreover, the leukocytes present in the blood of patients exhibited higher frequency of CD4+CD25+ T cells expressing CTLA-4, GITR, membrane-bound TGF-β, and Foxp3 and stronger ability to suppress the proliferation of allogeneic T cells when compared with CD4+CD25+ T cells of controls subjects. These data suggest that besides accumulating in the lesion sites caused by the fungal infection, the CD4+CD25+ T cells have the potential to regulate the effector function of APC and T cells.

Materials and Methods

**Patients with chronic PCM and healthy control subjects**

Leukocytes from blood obtained from 34 untreated patients recently diagnosed with an active chronic form of PCM (all men; age range: 21–71 years) were enrolled in this study. All patients exhibited clinical and laboratory features of the disease, such as lung infiltrates, polyadenopathy, or laboratory features of the disease, such as lung infiltrates, polyadenopathy, and/or skin lesions and specific Abs. The diagnosis of disease was corroborated by clinical specimens and serology at the Clinical Hospital of School of Medicine (Ribeirão Preto, University of São Paulo, São Paulo, Brazil). Twenty-one patients with lesions affecting the mucosal cavity and/or skin were submitted to biopsy. Patients with coinfections were not included in this study. Blood sample from 12 healthy volunteers with no history of pulmonary disease and with a negative result to paracoccidioidin skin test were enrolled in the study. Control biopsy samples were obtained from a woman undergoing cosmetic breast surgery.

The experimental protocol used was approved by Institutional Review Board of School of Medicine (Ribeirão Preto). Informed consent was obtained from all subjects before performing the studies.

**Media and reagents**

All human cells were grown in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 10 mM HEPES, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (all from Sigma-Aldrich). PHA and PE-conjugated streptavidin were purchased from Invitrogen Life Technologies. The recombinant human (h) IL-2 was obtained from BD Biosciences.

**Abs and flow cytometry analysis**

For immunostaining, PerCP, PE-, and FITC-conjugated Abs against CD3 (UCHT 1), CD4 (RPA-T4), CD8 (RPA-T8), CD14 (M5E2), CD19 (HI149), and CD1a (HI149), CCR4 (205410), and the respective mouse and rat isotype controls were used (BD Biosciences). PE-conjugated mice mAb anti-human GITR (110416), anti-CCR5 (C5CS), and biotinylated anti-TGF-β1 (LAP-27240) were purchased from R&D Systems. PE-conjugated anti-CD103 (Ber-ACT8) and purified anti-Foxp3 were purchased from eBioscience and Abcam, respectively. The Abs used for intracellular cytokine staining were PE-conjugated anti-IL-10 (JES3-19F1), anti-IFN-γ (4S.B3) (BD Biosciences), and biotinylated anti-TGF-β3 (4492; R&D Systems). The cell acquisition was performed on a FACSort flow cytometer using and CellQuest software (BD Biosciences). Unconjugated anti-CD3 (UCHT 1) and anti-CD28 (CD28.2) (BD Biosciences) were used for polyclonal activation and anti-human CCL5, CCL17, CCL22 (Santa Cruz Biotechnology), and Foxp3 (Abcam) were used for immunohistochemistry assays.

**Isolation of leukocytes**

Peripheral blood was harvested with heparin (50 U/ml) from healthy subjects and PCM patients. PBMC were isolated using Ficoll-Hypaque (Pharmacia Biotech) density gradient centrifugation, washed, counted, and labeled with specific Abs for phenotypic analysis in flow cytometer or for purification of T cell subpopulations. To characterize the leukocytes present in the lesion site, the biopsies of skin and mucosal lesions from patients were collected and incubated 1 h at 37°C in RPMI 1640 medium, containing 50 μg/ml liberase CI enzyme blend (Boehringer Ingelheim Chemicals). Next, the tissue were dissociated, for 4 min, in the presence of RPMI 1640 with 10% serum and 0.05% DNase (Sigma-Aldrich) using a Dounce homogenizer (BD Biosciences). The leukocytes viability was evaluated by trypan blue exclusion and used for cell activation or immunolabeling assays.

**T cell stimulation**

CD4+ T cells were isolated from PBMC by negative selection using magnetic beads (Miltenyi Biotec). CD4+CD25+ T cells were isolated from the pure, untouched CD4+ T cells using CD25 microbeads (Miltenyi Biotec). Due to the low yield of pure cells, the CD4+CD25+ were cultured in 96-well tissue-culture U-bottom plates (Corning) and primarily activated with anti-CD3 mAb (0.5 μg/ml) in the presence of anti-CD28 (1 μg/ml) and PHA (1 μg/ml). rHL-2 (10 ng/ml) was added at days 2, 5, and 7 after primary stimulation. At day 10, the cells were harvested and used in a secondary anti-CD3 and PHA stimulation with identical conditions. At day 15, after secondary stimulation, cocultures of CD4+CD25+ T cells were performed to analyze the influence of these cells on the proliferation assays with allogeneic lymphocytes.

**Cocultures and proliferation assays**

To verify the regulatory function of CD4+CD25+ T cells isolated from lesions or PBMC, they were cultured with PBMC (1×10^6/well) from normal donors, at ratio 1:10, in 96-well U-bottom plates, in presence of PHA (1 μg/ml), at 37°C and 5% CO2. On day 4, 0.5 μg/ml [H]thymidine (Amersham Biosciences) was added for the final 16 h of culture performed in triplicate. Incorporation of [H]thymidine was measured using a liquid scintillation counter (Beckman Instruments). The stimulation index (SI) was calculated based on the PHA-induced cell proliferation in the presence or not of CD4+CD25+ T cells divided by the basal proliferation. The data expressed as percentage of inhibition were calculated based on the PHA-induced proliferation of allogeneic T cells cultured without CD4+CD25+ T cells.

**Intracellular cytokines and Foxp3 detection**

The intracellular detection of IL-10, TGF-β, IFN-γ, and Foxp3 in leukocytes obtained from lesions and blood (for Foxp3) of patients was performed in fixed and permeabilized cells using Cytofix/Cytoperm (BD Biosciences). First, the cells were labeled with Abs of cell surface such as PerCP-conjugated anti-CD25 and PerCP-conjugated anti-CD4. The cells were fixed, permeabilized, stained with PE-labeled anti-human (h) IL-10, PE-labeled anti-hIFN-γ, biotin-labeled anti-tTGF-β, incubated with PE-streptavidin (from Invitrogen Life Technologies) or control isotype, and analyzed using a FACS. The graphs represent the absolute number of cells/biopsy, percentage of cells or mean fluorescence intensity (MFI). The absolute numbers of leukocyte/biopsy subsets were calculated through percentage obtained by FACS and the amount of cells were determined in Neubauer chamber. To Foxp3 detection, the leukocytes from blood and lesions were stained with rabbit polyclonal to Foxp3 that reacts with human (Abcam) and biotinylated anti-rabbit (Vector Laboratories) followed by PE-streptavidin according to the manufacturer’s protocol.

**Cytokine assays**

Cytokine concentrations in supernatants of coculture assays from PBMC of patients were determined using a Cytokine Bead Array kit (BD Cytometric Bead Array) according to the manufacturer’s instructions (BD Biosciences).

**Immunohistochemical analysis**

All human cells were grown in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 10 mM HEPES, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (all from Sigma-Aldrich). PHA and PE-conjugated streptavidin were purchased from Invitrogen Life Technologies. The recombinant human (h) IL-2 was obtained from BD Biosciences.
Statistical analysis

Data obtained from flow cytometry and cells proliferation assay were expressed as the SEM. Statistical analysis was performed using ordinary ANOVA followed by the Tukey’s multiple comparison test (INSTAT Software; GraphPad). All values were considered significantly different at $p < 0.05$.

Results

**Phenotypic characterization of CD4$^+$CD25$^+$ T cells in PBMC from patients with PCM**

First, we analyzed the phenotype of circulating subpopulations of lymphocytes (gate R1), as well as monocytes and dendritic cells (DC) (gate R2) in PBMC from patients with the chronic form of PCM and healthy controls subjects (Fig. 1A). We found that the frequencies of cells expressing CD19$^+$, CD3$^+$CD4$^+$, CD3$^+$CD8$^+$, CD4$^+$CD25$^+$, and CD14$^+$ in patients with PCM were similar to those detected in normal donors. Conversely, the frequency of CD1a$^+$CD11c$^+$ cells in the blood of patients was increased ($p < 0.05$) compared with the controls (Fig. 1B). The staining of the cell surface of CD3$^+$CD4$^+$ T cells in peripheral blood of 28 patients revealed that 3.5% of them also expressed the IL-2R$\gamma$-chain CD25 (Fig. 1, B and E). Moreover, CD25$^{\text{high}}$ cells represent 1.1% of the total cells (gate R1) and 23.9% of the

**FIGURE 1.** Characterization of CD4$^+$CD25$^+$ Treg cells in control subjects and patients with PCM. A, PBMC from control (□) and patients (■) in these analyses were gated on lymphocytes (R1), and monocytes/DCs (R2) via their forward (FSC) and side scatter (SSC) properties. B, The CD19$^+$, CD3$^+$CD4$^+$, CD3$^+$CD8$^+$, CD4$^+$CD25$^+$, CD14$^+$, and CD11c$^+$CD1a$^+$ cells on freshly isolated PBMCs from both groups was initially evaluated by flow cytometry. The inset in B shows the percentage of CD4$^+$ T cells expressing very high levels of CD25 (CD25$^{\text{high}}$) is indicated. The representative flow cytometry analysis of CD4$^+$CD25$^+$, CD4$^+$CD25$^{\text{high}}$, and CD4$^+$CD25$^-$ gated lymphocytes (R1) is shown in E. The CD4$^+$CD25$^+$(C), CD4$^+$CD25$^{\text{high}}$ (D), and CD4$^+$CD25$^-$ (F) cells were analyzed for their expression of membrane CTLA-4, GITR, CD103, CD45RO, CCR5, CCR4, LAP, and intracellular Foxp3. The results are expressed as the mean ± SEM for patients ($n = 28$) or control subjects ($n = 12$) tested individually. ***, $p < 0.001$; **, $p < 0.01$; and *, $p < 0.05$ compared with controls.
CD4+CD25+ T cells (inset in Fig. 1B). Similar data were found in the controls. Because it cannot be properly excluded that the CD4+CD25+ T cell subset also contains conventional activated T cells, in addition to Treg cells, we next evaluated the expression of surface molecules CTLA-4, GITR, CD103, CD45RO, CCR5, CCR4, latency associated peptide (LAP), and Foxp3 in these cells and the CD4+ T cells expressing low and high levels of CD25. The population of CD4+CD25+ cells expressing CD103, CD45RO, and the chemokines receptors CCR5 and CCR4 was similar to that found in healthy controls. However, the expression of CTLA-4 (19.3 ± 5.0%), GITR (25.0 ± 8.0%), Foxp3 (7.8 ± 1.4%), and LAP (57.8 ± 11.2%) in PBMC of patients was higher (p < 0.05) than that found in PBMC of control subjects (2.6 ± 0.9%, 6.1 ± 2.9%, 1.9 ± 1.1%, 19.7 ± 5.5% for CTLA-4, GITR, Foxp3, and LAP, respectively) (Fig. 1C). In addition, low frequency of CD4+CD25low expressed CTLA-4 (6.9 ± 2.6%), GITR (14.3 ± 6.9%), and Foxp3 (3.9 ± 3.9%), and no differences were found in patients and controls in this subpopulation (data not shown). Notably, high levels of Treg cell markers were observed in the CD4+CD25high, and patients presented a significant increased of CTLA-4 and Foxp3 expression in this subset (Fig. 1D). The prominent difference in the CD4+CD25+GITR+ T cells in patients and controls was observed in the CD4+ T cells expressing medium levels of CD25. On the contrary, no difference was noted in the subpopulation of CD4+CD25+ T cells from patients and control expressing CTLA-4, GITR, CD103, CCR5, Foxp3, and LAP (Fig. 1F).

Functional characterization of CD4+CD25+ T cells of PBMC from patients with PCM

We next studied whether the higher frequency of CD4+CD25+ T cell expressing CTLA-4, GITR, Foxp3, and LAP in patients is correlated with their regulatory properties. Therefore, the CD4+CD25+ and CD4+CD25− T cells from patients and healthy controls were immunomagnetically sorted, expanded, cultured with allogeneic T cells (ratio of 1:10), and the proliferative response was evaluated. The SI of the allogeneic T cells cultured with PHA was similar in the presence of CD4+CD25+ T cells from patients or controls (Fig. 2A). Conversely, the addition of CD4+CD25+ T cells significantly inhibited the T cell proliferation. Interestingly, the inhibition induced by CD4+CD25+ T cells from patients was significantly higher than that observed with CD4+CD25+ T cells from control subjects (Fig. 2A). In addition, the CD4+CD25+ T cells from patients inhibited the production of IFN-γ (Fig. 2B) and TNF-α (Fig. 2C) (p < 0.05), but not IL-10 (Fig. 2D), and low levels of IL-2 production were detected in cocultures (<7 pg/ml). These data clearly indicate that the CD4+CD25+ T cells are in fact active Treg cells.

The CD4+CD25+ T cells migrate to the lesions induced by fungal infection

We next determined the phenotype of leukocytes in mucosal or skin lesions obtained from 15 patients with PCM. CD3+ cells represent 48.2 ± 5.2% of the cells infiltrate, being that 3.8 × 105 (range from 0.2 to 8.5 × 105) cells/biopsy were CD3+CD4+, 3.1 × 105 (range from 1.0 to 6.0 × 105) cells/biopsy were CD3+CD8+. The CD4+ T cells that coexpressed CD25 ranged from 6.0 × 102 to 2.8 × 103 cells/biopsy (7.0 ± 1.2%). In addition, CD11b+CD11c+ (macrophages) and CD11c+CD1a+ (DCs) were also identified in the lesions (Fig. 3A). Gating on CD4+CD25+ T cells (Fig. 3B), we found that they express CTLA-4 (56.5 ± 7.9%), GITR (68.7 ± 11.7%), CD103 (23.1 ± 7.8%), CD45RO (85.6 ± 6.3%), and the chemokine receptors CCR5 (63.9 ± 9.2%) and CCR4 (70.5 ± 9.2%) (Fig. 3C). Representative histogram figures showed that 42.6% of the CD4+CD25+ T cells expressed Foxp3 (Fig. 3E) and 12.1% expressed LAP (Fig. 3F). Significantly lower expression of CTLA-4, GITR, CD103, CCR5, CCR4 (Fig. 3D), Foxp3 (Fig. 3G), and LAP...
lyzed on CD4/H11001 CD4 isotype-matched control. The positivity cells were determined over the CD4 \textsuperscript{+}CD25\textsuperscript{+} (C) and CD4 \textsuperscript{+}CD25\textsuperscript{−} (D) T cells were analyzed for their expression of CTLA-4, GITR, CD103, CD45RO, CCR5, and CCR4. The results are expressed as the mean ± SEM for patients (n = 15). The expression of Foxp3 (E and G) and surface-bound TGF-β1 LAP (F and H) was analyzed on CD4 \textsuperscript{+}CD25\textsuperscript{+} and CD4 \textsuperscript{+}CD25\textsuperscript{−} gated T cells. The empty histograms represent the appropriated isotype-matched control and the full histograms the anti-Foxp3 and anti-LAP. The result is representative of one of four biopsies analyzed.

The expression of chemokines in P. brasiliensis-induced lesions

It was important to know the mechanism that could guide the CD4 \textsuperscript{+}CD25\textsuperscript{+} T cells to the lesions. Because CD4 \textsuperscript{+}CD25\textsuperscript{+} T cells expressing CCR4 migrate to a microenvironment containing CCL17 and CCL22 (24, 25) and 71% of them in the lesions from patients are CCR4\textsuperscript{+}, we looked for the CCR4 ligands secreted in the biopsies. In these lesions, we observed a well-defined granulomatous reaction circumscribed with aggregates of macrophages, epithelioid, and multinucleated giant cells, lymphocytes, and polymorphonuclear cells, surrounding the fungal yeast forms (Fig. 4A). By immunohistochemistry, we detected inflammatory cells expressing CCL17 (Fig. 4C), CCL22 (Fig. 4D), and CCL5 (Fig. 4E). The controls, done in the absence of secondary Ab (Fig. 4B) or normal skin (data not shown), were always negative. The expression of chemokines ligands CCR4 and CCR5 may guide the CD4 \textsuperscript{+}CD25\textsuperscript{+} T cells to P. brasiliensis-induced lesions.

The expression of cytokines by CD4 \textsuperscript{+}CD25\textsuperscript{+} and CD4 \textsuperscript{+}CD25\textsuperscript{−} T cells from the lesions of patients with PCM

Next, we evaluated the profile of intracellular cytokine in CD4 \textsuperscript{+}CD25\textsuperscript{+} and CD4 \textsuperscript{+}CD25\textsuperscript{−} T cells isolated from biopsies of patients with PCM. The data showed that the numbers of CD4 \textsuperscript{+}CD25\textsuperscript{−} T cells expressing IL-10 and IFN-γ were similar to CD4 \textsuperscript{+}CD25\textsuperscript{+} T cells (Fig. 5A). Differently, the number of CD4 \textsuperscript{+}CD25\textsuperscript{+} T cells producing TGF-β was significantly higher than their CD4 \textsuperscript{+}CD25\textsuperscript{−} counterparts, with an MFI of 67.7 ± 21.9 vs 15.9 ± 8.2 (Fig. 5, A and B). With regard to the percentage of cells found in the biopsies, the CD4 \textsuperscript{+}CD25\textsuperscript{+} T cells positives for IL-10, TGF-β, and IFN-γ were significant higher when compared with CD4 \textsuperscript{+}CD25\textsuperscript{−} T cells (Fig. 5C).

Functional characterization of CD4 \textsuperscript{+}CD25\textsuperscript{+} T cells from lesions of patients with PCM

Because CD4 \textsuperscript{+}CD25\textsuperscript{+} T cells are present in the lesions, the leukocytes isolated from five patients (P1–P5), tested individually, were expanded, sorted, and tested for their ability to suppress the proliferation of allogeneic PBMC. The SI of allogeneic T cells cultured with PHA was 30.1 ± 10. Similar values were found when expanded CD4 \textsuperscript{+}CD25\textsuperscript{−} T cells (1 × 10\textsuperscript{4} cells/well) isolated from lesions were added in these cultures (SI = 40.6 ± 8.5). However, the addition of expanded CD4 \textsuperscript{+}CD25\textsuperscript{+} T cells from patients (1 × 10\textsuperscript{4} cell/well) to allogeneic cells (1 × 10\textsuperscript{5} cell/well) significantly suppressed the T proliferation induced by PHA (Fig. 6). These findings show that functional Treg cells accumulate in the lesions of patients with the chronic form of PCM.

Discussion

CD4 \textsuperscript{+}CD25\textsuperscript{+} cells are known to suppress the activation and effector functions of T cells (15, 18, 19, 22) by secreting IL-2 (26, 27), the production of TGF-β (15, 16, 20, 22), and by displaying CTLA-4 on the cell membrane (28). PBMC from patients with PCM are also capable of suppressing the effector functions of T cells. PBMC from these patients also express CTLA-4 and FasL.
but inhibition of these molecules only partially restores IFN-γ production and the proliferation of T cells in response to PHA (8). The latter studies suggested that suppressive mechanisms akin to those of CD4<sup>+</sup>CD25<sup>+</sup> Treg-mediated cell unresponsiveness in PCM patients. In the present study, we demonstrate that the total number of CD4<sup>+</sup>CD25<sup>+</sup> T cells is similar in healthy individuals and patients with PCM. However, CD4<sup>+</sup>CD25<sup>+</sup> T cells with a regulatory phenotype are elevated both in blood and in the lesion of patients. Moreover, we show that these cells are functionally active and capable of inducing T cell unresponsiveness, as observed in PCM patients.

The relative number of leukocytes in the blood of PCM patients was not different from that of healthy individuals, except for an increase in the number of CD1a<sup>+</sup>CD11c<sup>+</sup> DC. Although we cannot explain the reason for this increase, it is possible that the fungal infection and the Ags released in the circulation (29) may promote

**FIGURE 4.** CCL17, CCL22, CCL5, and Foxp3 expression in the lesions of patients with PCM. Tissue sections were obtained from biopsies of eight patients with PCM, fixed in acetone, and the morphology (A), CCL17 (C), CCL22 (D), CCL5 (E), and Foxp3 (F) expression was determined. A, The characteristic granulomatous reaction found in chronic patients. For the immunohistochemistry, the tissue were incubated with IgG1 isotype control (B), goat anti-human CCL17 (C), CCL22 (D), CCL5 (E), or Foxp3 (F), followed by biotinylated mouse anti-goat IgG and avidin biotin peroxidase. The diaminobenzidine (DAB) was used as the peroxidase substrate to generate a brown-staining signal and counterstained with hematoxylin. Magnification, ×200 for figures A, B, C, F, and ×400 for E.

**FIGURE 5.** Cytokine profiles of CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cell subsets in the lesions of patients with PCM. The leukocytes isolated from lesions were analyzed by flow cytometry for their intracellular IL-10, IFN-γ, and TGF-β expression at the CD4<sup>+</sup>CD25<sup>+</sup> (■) or CD4<sup>+</sup>CD25<sup>-</sup> (□) T cells. The bar graph represents the mean ± SEM of absolute numbers (A), the MFI (B), and the percentage (C) of CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cells obtained per biopsy (n = 4). *, p < 0.05 and *** p < 0.001, compared with CD4<sup>+</sup>CD25<sup>-</sup> T cells.

**FIGURE 6.** Functional characterization of CD4<sup>+</sup>CD25<sup>+</sup> T cells derived from lesions of patients with PCM. Freshly allogeneic PBMC (1 × 10<sup>5</sup> cells/well) were stimulated with PHA in the presence or not of magnetic bead-sorted expanded CD4<sup>+</sup>CD25<sup>+</sup> T cells (1 × 10<sup>4</sup> cells/well) isolated from lesions of patients (P) with PCM (n = 5, P1–P5). Proliferation was determined after 4 days of culture by the addition of [3H]thymidine incorporation for the last 16 h. The bar graphs (mean ± SEM) indicate the percentage of suppression of proliferation in the presence of CD4<sup>+</sup>CD25<sup>+</sup> T cells from patients at a ratio of 1:10, calculated based on the PHA-mediated proliferation in allogeneic T cells cultured without CD4<sup>+</sup>CD25<sup>+</sup> T cells. The mean ± SEM of the SI of allogeneic T cells and CD4<sup>+</sup>CD25<sup>+</sup> T cells (1 × 10<sup>4</sup> cells/well) cultured with PHA were 30.1 ± 10.1 and 40.6 ± 8.5, respectively.
the mobilization of DC into the peripheral circulation and tissues (30). We did not confirm that the ratio of CD4+CD8 was decreased in patients with PCM (31). The latter results may reflect the fact that patients studied here exhibited the chronic form of disease. The number of CD4+ T cells coexpressing CD25 and CD25<sup>high</sup> was also similar in both groups studied. However, the expression of CTLA-4, GITR, membrane-bound TGF-β, and intracellular Foxp3, characteristic markers of natural T cells (15, 16, 19–21, 28), was increased in CD4+CD25<sup>+</sup> T cells from patients as compared with healthy individuals. In addition, the CD4+CD25<sup>high</sup> T cells isolated from patients expressed greater levels of Foxp3 and CTLA-4 while the CD4+CD25<sup>low</sup> T cell subset expressed lower levels of these markers, supporting the idea that this subset is related with activated T cells. Our present data revealed an increase in the number of cells with a phenotype characteristic of natural Treg cells in the blood of PCM patients.

Natural Treg cells are Foxp3<sup>+</sup> and belong to the CD4+CD25<sup>high</sup> T cell subpopulation with marked suppressive capacity (32, 33). Consistently with their natural Treg cell phenotype, expanded CD4+CD25<sup>+</sup> T cells but not the expanded CD4+CD25<sup>−</sup> T cells from PCM patients suppressed PHA-induced allogeneic T cell proliferation (Figs. 1 and 2). The suppression of the PHA-induced IL-2, TNF-α, and IFN-γ, but not IL-10 production by the added CD4+CD25<sup>+</sup> T cells is also characteristic of Treg cell function (16, 17). The absence of IL-2 in cultures of cells from patients may be a consequence of Treg cell maintenance, acting as an "IL-2 sink" and then inhibiting other T cell responses solely by consuming IL-2 (27). The mechanisms that lead to increased expression of Foxp3 and CTLA-4 and, consequently, higher suppressive activity or the Ag specificity of CD4+CD25<sup>+</sup> T cells from patients is not known. It is possible that fungal products mediate this response because Ag recognition by natural Treg cells promotes their expansion and increases their regulatory function (18, 34). Thus, it is clear that cells with a natural Treg cell phenotype were found in blood of patients with PCM and could inhibit T cell responses.

Because natural Treg cells preferentially accumulate at sites of infection, limiting the effector immune responses and promoting pathogen survival (18, 23, 34), we investigated their presence in the lesions of patients with PCM. We found that CD4+CD25<sup>+</sup> T cells expressing CTLA-4, GITR, CD45RO, LAP, and Foxp3 were present in P. brasilensis-induced lesions. Aside from these markers, the majority of CD4+CD25<sup>+</sup> T cells also expressed CD103, CCR4, and CCR5, all of which are involved in T cell migration (24, 25, 35–37). Therefore, the increased presence of CCL17, CCL22 (CCL4 ligands), and the CCL5 (CCR5 ligand) (Fig. 4), CCL3, and CCL4 (our unpublished observations) in the skin lesions of PCM patients may promote CD4+CD25<sup>+</sup> T cell migration into peripheral sites. It is possible that the pathogens, such as P. brasilensis, may have evolved strategies to establish conditions favoring natural T cell recruitment into sites of infection, through triggering the secretion of CCR4- and CCR5-acting chemokines by infected macrophages (38). However, CCR4 is also crucial for homing of Th cells and Th2, mainly memory T cells, into inflamed skin (39). In fact, we found CD4+CD25<sup>+</sup> T cells in the blood and lesions coexpressing CCR4 and CD45RO, a phenotype related to memory T cells (40).

Other interesting results pointed to differential cytokine generation by T cell populations from PCM patients. Our results also showed that a significant number of CD4+CD25<sup>+</sup> T cells in patient lesions expressed high levels of intracellular and membrane-bound TGF-β. This cytokine is strongly associated with the suppression of proinflammatory responses, the faster growth of parasites, and the presence and expansion of CD4+ T cells with the hallmarks of Treg cells (18, 41). Moreover, TGF-β and IL-10 and parasite Ags create a favorable environment that may promote the survival of Treg cells in the site of infection (18). It was recently demonstrated that natural Treg cells from chronically infected mice produce IL-10 in response to Leishmania major parasite Ags (34) and, in some cases, is associated with Treg function in experimental models. However, in humans, most in vitro results have showed a relative contribution of cell-cell contact and TGF-β in the Treg suppressive functions (42). From this study, both CD4+CD25<sup>+</sup> and CD4+CD25<sup>−</sup> T cells from the lesions of patients expressed IL-10. These data suggest that distinct subsets of CD4+ T cells with regulatory properties are present in P. brasilensis-induced lesions (i.e., IL-10-producer T cells, Tr1). Although we did not define the specificity of production IL-10 by CD4+CD25<sup>+</sup> T cells as well as their role in the Treg cell function, this immunoregulatory cytokine has been described to play an important role in impairment of the cell-mediated immune response of patients with PCM (9, 12, 13). Indeed, the addition of anti-IL-10 partially restored T cell proliferation and IFN-γ production (43). IFN-γ-producing cells, ~37% of CD4+CD25<sup>+</sup> T cells, were present in PCM lesions. These cells could be related with recently activated Th cell populations, because some of them did not express characteristic markers of Treg cells, including CTLA-4 and Foxp3. Furthermore, we detected the production of TGF-β, IL-10, and IFN-γ by freshly isolated CD4+CD25<sup>+</sup> T cells suggesting that fungal Ags could activate them. Collectively, these data indicate that Treg cells found in chronic lesions must be involved in the modulation of local immune response. However, whether Treg cells are associated with pathology for increasing tissue damage remains to be determined.

Indeed, we found that CD4+CD25<sup>+</sup> T cells from chronic, but not acute, PCM patients inhibited the allogeneic proliferative response mediated by PHA (our unpublished observation). Unfortunately, due to size of biopsies, a limited number of cells were obtained, and it was not possible to perform experiments to identify the possible phenotypic changes to define Treg cells from acute and chronic PCM patients used in cocultures assays. We plan to test this hypothesis in future experiments because natural Treg cells change their phenotype and are more suppressive with the progress of the chronic stage of experimental schistosomiasis (44).

These data, together with the higher expression of regulatory soluble factors, Foxp3, CTLA-4, and bound-membrane TGF-β expression, strongly indicate that CD4*CD25* T cells found in lesions may be effectively contributing to the regulation of the effector local immune response during the chronic phase of PCM disease. The regulation of local responses could contribute to the maintenance of the fungus in the chronic lesions. Indeed, yeast cells were found in all biopsies studied (data not shown). In agreement with our findings, the CD4*CD25* T cells in L. major-induced skin lesion (18, 23, 34, 35), S. mansoni egg-induced granulomas (44), and Candida albicans (45) control effector immune response, allowing pathogen persistence. Thus, the enhanced expression and function of cells with a natural Treg phenotype in lesions of patients with PCM suggest this cell type to be a major contributor to parasite persistent and induction of chronicity.

Thus, we demonstrate that natural Treg cells defined by their phenotype and function are present in blood and lesions of patients with the chronic form of PCM, suggesting these cells may control systemic and local immune response in this disease. Further studies are, however, required to understand the relationship between Treg, effector T cell activation, and the mechanisms underlying the maintenance of P. brasilensis or disease reactivation. We believe this study is a necessary step toward the design of future therapeutic protocols needed to treat chronic inflammatory diseases and, particularly, chronic PCM infection.
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Disclosures
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