Increased Expression of Regulatory T Cells and Down-Regulatory Molecules in Lepromatous Leprosy

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Abstract. T regulatory cells (Tregs) play an important role in the mechanism of host's failure to control pathogen dissemination in severe forms of different chronic granulomatous diseases, but their role in leprosy has not yet been elucidated; 28 newly diagnosed patients (16 patients with lepromatous leprosy and 12 patients with tuberculoid leprosy) and 6 healthy *Mycobacterium leprae*-exposed individuals (contacts) were studied. Tregs were quantified by flow cytometry (CD4+ CD25+ Foxp3+) in peripheral blood mononuclear cells stimulated *in vitro* with a *M. leprae* antigenic preparation and phytohemagglutinin as well as in skin lesions by immunohistochemistry. The lymphoproliferative (LPR), interleukin-10 (IL-10), and interferon-γ (IFN-γ) responses of the *in vitro*-stimulated peripheral blood mononuclear cells and the *in situ* expression of IL-10, transforming growth factor-β (TGF-β), and cytotoxic T-lymphocyte antigen 4 (CTLA-4) were also determined. We show that *M. leprae* antigens induced significantly lower LPR but significantly higher Treg numbers in lepromatous than tuberculoid patients and contacts. Mitogen-induced LPR and Treg frequencies were not significantly different among the three groups. Tregs were also more frequent *in situ* in lepromatous patients, and this finding was paralleled by increased expression of the antiinflammatory molecules IL-10 and CTLA-4 but not TGF-β. In lepromatous patients, Tregs were intermingled with vacuolized hysticote infiltrates all over the lesion, whereas in tuberculoid patients, Tregs were rare. Our results suggest that Tregs are present in increased numbers, and they may have a pathogenic role in leprosy patients harboring uncontrolled bacillary multiplication but not in those individuals capable of limiting *M. leprae* growth.

INTRODUCTION

A spectrum of different clinical, bacteriologic, and histological manifestations is described in leprosy patients, which correlates to variations in host immune responses.[1] In patients with tuberculoid leprosy, the histological findings are characterized by infiltration of CD4+ T lymphocytes, compact granulomas, and few or no detectable bacilli. *In vitro*, such patients show vigorous lymphocyte proliferative responses and a Th-1 cytokine profile. In the opposite pole, lepromatous leprosy reveals a poor granulomatous response, with few infiltrating CD4+ T cells, numerous bacilli, decreased Th-1 immune response, and shift to a Th-2 profile.[2] *In vitro*, these findings are translated into lack of *Mycobacterium leprae*-specific lymphoproliferative responses.[3]

Recently a subset of CD4+ T cells with suppressive function has been characterized[4]; these cells are best identified because of constitutive expression of CD25, the α-chain of the interleukin-2 (IL-2) receptor, and the forkhead/winged helix transcription factor 3 (FoxP3). The mechanisms involved in suppressing effector CD4+ T cells are still a subject of intense study, and several non-excluding mechanisms have been described.[5] These mechanisms can be mediated through either direct interaction between T regulatory cells (Tregs) and effector T cells or the release of cytokines. Tregs express high levels of CTLA-4, a costimulatory molecule that suppresses several functions of CD4+ T cells such as proliferation and IL-2 secretion.[4,5] Tregs also have the ability to secrete high amounts of the antiinflammatory cytokines IL-10 and trans-

forming growth factor-β (TGF-β).[4,5] Both experimental and human studies have shown that autoimmune disorders like lupus and systemic sclerosis are associated with Treg depletion both *in situ* and in peripheral blood.[6–8] However, it has also been shown that Tregs play a crucial role in the regulation of the immune response to microbial antigens and may favor the persistence of pathogens causing chronic granulomatous disease such as leishmaniasis and tuberculosis.[9–11]

Although Tregs exert an important role in the mechanism of host's failure to control pathogen dissemination in different chronic granulomatous diseases, their role in leprosy has not yet been elucidated.

PATIENTS AND MATERIALS

Patients and controls. A total of 28 consecutive newly diagnosed leprosy patients admitted to our service were analyzed. All patients were studied before start of specific therapy; they were all human immunodeficiency virus (HIV)-negative and without other infectious comorbidities; 16 patients had lepromatous leprosy (age range = 38 ± 4 years; 8 female and 8 male subjects), of which 13 patients had borderline lepromatous leprosy (BL) and 3 patients had polar lepromatous leprosy (LL). The other 12 patients had tuberculoid leprosy (age range = 45 ± 5 years; 7 female and 5 male subjects), of which 10 patients had the borderline form (BT) and 2 patients had the polar form (TT). The first group had multibacillary disease, whereas the second group had a paucibacillary disease. A control group was made of six healthy exposed individuals (contacts; 35 ± 4 years) selected among persons living in close contact with the lepromatous leprosy patients. Exposure was defined by a positive response in the lymphoproliferation assay with the *M. leprae* antigen described below (Figure 1B). The study was approved by the Ethics Committee of the

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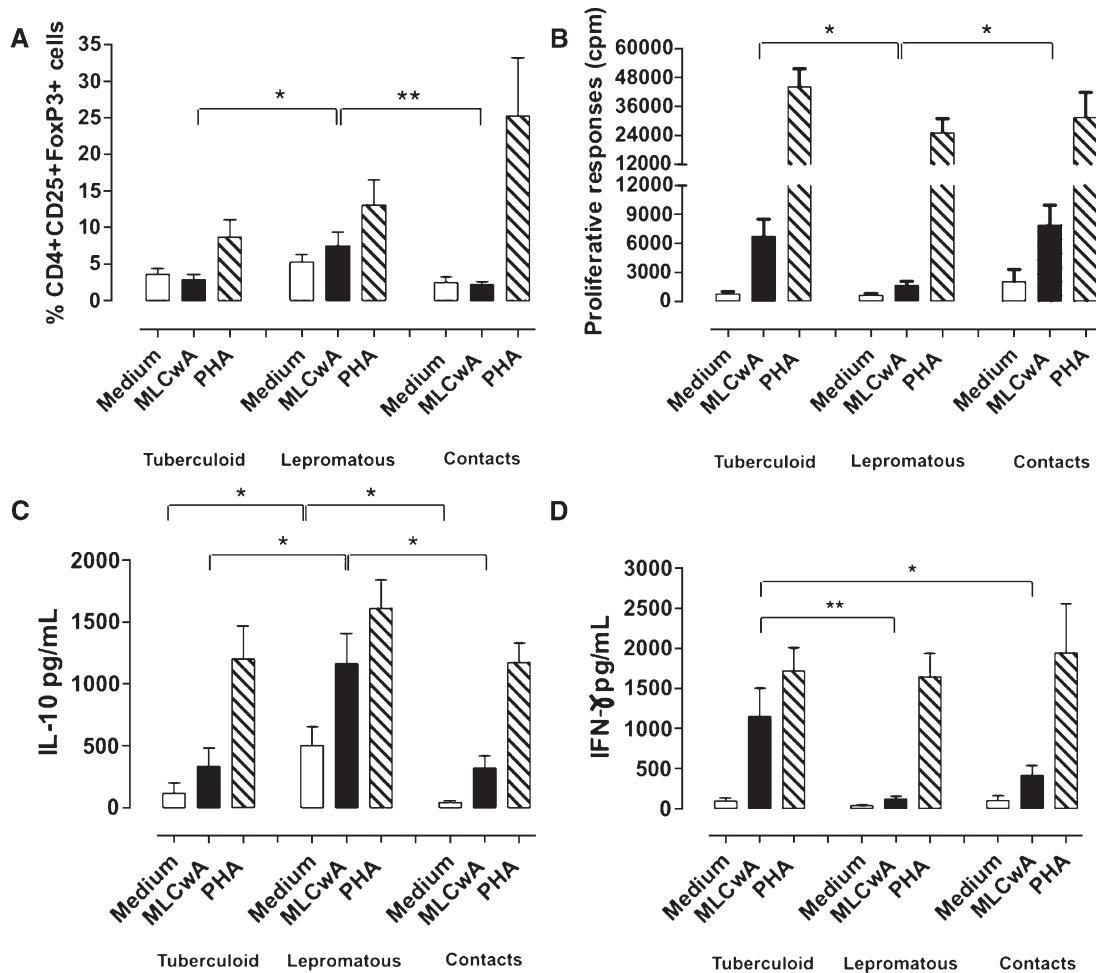†These authors are senior authors.

FIGURE 1. Treg (CD4+ CD25+ Foxp3+) frequency (**A**), LPR (**B**), IL-10 secretion (**C**), and IFN-γ secretion (**D**) in 2.5×10^5 PBMCs of tuberculoid ($N = 10–12$), and lepromatous ($N = 14–16$) leprosy patients and healthy exposed contacts ($N = 6$) stimulated *in vitro* with phytohemagglutinnin (PHA) or a *M. leprae* antigen (MLCwA) or not stimulated (medium). Results are presented as mean ± SEM. *$P < 0.05$; **$P < 0.01$.

Clinics Hospital, University of São Paulo Medical School (#0955/08). Informed consent was obtained from all participants. Blood was collected from patients and contacts, whereas a biopsy was taken from a typical lesion of the patients; no biopsies could be taken of normal skin of the patients or contacts for ethical reasons.

Peripheral blood mononuclear cells immunoassays. Peripheral blood mononuclear cell (PBMC) cultures were carried out as previously described.[12] PBMCs were isolated from heparinized peripheral blood by density gradient and resuspended in RPMI supplemented with gentamicin (40 μg/mL) and 10% human AB serum (Sigma-Aldrich, St. Louis, MO). PBMCs were cultivated in 96- (1.0×10^5/well) and 24- (1.0×10^6/well) well flat-bottomed plates with medium only, a cell wall preparation of *M. leprae* (MLCwA; 20 μg/mL), and as positive control, the mitogen phytohemaglutinin (PHA; 5 μg/mL; Sigma) at 37°C and 5% CO_2 as previously described.[9] For lymphoproliferation assays (LPR), cells were incubated for 3 (PHA) and 6 (MLCwA) days in triplicates in 96-well plates and pulsed for additional 18 hours with 1.0 μCi/well (^{3}H)thymidine (Radiochemical Center, Amersham, UK) before harvest. Cell-bound radioactivity was measured using a β-plate scintillation counter (Perkin Elmer, Boston, MA). Results were expressed as mean counts per minute of the triplicates.

For Treg quantification, the cultures were set out on 24-well plates. The cells were harvested at day 4, washed, and incubated with anti-CD4 (PC5; eBioscience, San Diego, CA) and anti-CD25 (FITC; Invitrogen, Camarillo, CA) for 20 minutes. The cells were then fixed and permeabilized (FIX & PERM; Invitrogen), and they were stained with anti-FoxP3 (PE; eBiociences). Cells were then washed and immediately acquired and analyzed by flow cytometry (Coulter Epics XL, Hialeah, FL). The expression of FoxP3 and CD25 was examined in the CD4+ lymphocytes gate according to the work by Sakaguchi and others.[4] Compensation of the populations analyzed was carried out in each experiment using unstained cells and the fluorescence minus one protocol.[13] The results were expressed as the percentage of FoxP3+ CD25+ cells per at least 10,000 CD4+ lymphocytes.

Enzyme-linked immunosorbent assay (ELISA) for quantification of interferon-γ (IFN-γ) and IL-10 secretion in supernatants was carried out as in previous studies using commercial kits (R&D Systems Inc., Minneapolis, MN) according to the manufacturer.[12] The supernatants were harvested from 24-well PBMC cultures at day 4 and stored in aliquots at –80°C until use. The lower limit of detection of the assays was 10 pg/mL.

Immunohistochemistry of lesion biopsies. Biopsies were taken with a standard dermatologic biopsy punch. Biopsies

were preferentially taken of well-circumscribed plaques in tuberculoid patients and in lepromatous patients, the most infiltrated lesions. For the immunohistochemical (IHC) study of patients' biopsies, a streptavidin-biotin peroxidase method was used.[14] Briefly, after deparaffinization and hydration, antigen recovery was performed in a retrieval solution at pH 9.0 (S2368; Dako, Carpinteria, CA) for 20 minutes at 95°C. The primary antibodies anti–IL-10 (MAB17; R&D), anti–TGF-β (Novocastra Leyca Biosystem, New Castle, UK), and anti–CTLA-4 (AF386-PB; R&D) as well as a labeled streptavidin–biotin complex (LSAB; Dako) were applied. 3,3-diaminobenzidine tetrahydroxychloride (DAB; Sigma) was used as chromogen, and the slides were counterstained with hematoxylin. All reactions were performed with positive and negative controls. The latter comprised isotype controls and omission of the primary antibody. Double immuno-staining reaction was used to detect Foxp3/CD25 coex-pression. Tissue sections were incubated with anti-Foxp3 antibody (14-4776; eBioscience) and developed with the LSAB complex (Dako). The peroxidase reaction was revealed with DAB/NiCl2 (Sigma). The second reaction was performed with the anti-CD25 antibody (Leyca Biosystem, New Castle, UK), and the reaction was developed with Envision alkaline phosphatase (K401; Dako) and Permanent Red chromogen (Dako). Quantification of immunostained cells was performed using AxioVision 4.8.2 software (Zeiss). Six images from each specimen were considered. The area of the granulomatous inflammatory infiltrate was measured, and quantification of positive cells was done exclusively in these areas by two independent observers (M.L.P. and C.P.) blinded to the patients' clinical status (lepromatous or tuber-culoid leprosy). Adjacent non-inflamed areas of the biopsies were also examined for the eventual presence of Tregs, which were not found. Ten biopsies of normal skin obtained during elective cardiac surgery of patients with coronary disease were used for standardizing the double immunostaining reac-tion as well as negative controls.

Statistical analysis. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's post-test for the immunoassays data and the Mann–Whitney test for the IHC data.

RESULTS

In vitro induction of Tregs (CD4+ CD25+ Foxp3+) expan-sion in PBMC cultures with PHA and MLCwA were evalu-ated in tuberculoid patients, lepromatous patients, and contacts (Figure 1A). There were significantly higher percent-ages of MLCwA-induced Tregs in lepromatous leprosy than tuberculoid patients and contacts. There was apparently no difference in the capacity of Treg expansion between the three groups, because the PHA-induced Tregs frequencies were not significantly different. To address the functional relevance of the observed higher proportion of Tregs in lepro-matous patients, we investigated two major phenotypic markers that characterize *in vitro* the anergic pole of leprosy, namely the high production of IL-10 and the lack of *M. leprae* antigens-specific lymphocyte proliferative responses.[2,3] In fact, lymphocytes from tuberculoid patients and contacts presented vigorous proliferative responses to PHA and MLCwA, whereas cells from lepromatous patients were only able to proliferate to PHA (Figure 1B). Significantly higher responses to MLCwA were found in tuberculoid patients and

contacts than lepromatous patients, whereas PHA responses did not show significant differences. Conversely, both unstimulated and MLCwA-stimulated PBMCs from leproma-tous patients released significantly more IL-10 than those PBMCs from tuberculoid patients and contacts, whereas release on PHA stimulation was not significantly different among the three groups (Figure 1C). We additionally deter-mined the IFN-γ level in these supernatants. As expected, IFN-γ levels were significantly lower in MLCwA-stimulated cultures from lepromatous patients than tuberculoid patients and contacts; those levels in PHA-stimulated cultures were again equivalent (Figure 1D). Thus, the increased expression of Treg cells in lepromatous patients coincided with deficient antigen-specific INF-γ and lymphocyte proliferative responses but increased IL-10.

To further assess the role of Tregs in leprosy patients, their frequency was investigated in the lesions of the patients. Biopsies of lesions were available from most tuberculoid ($N = 9$; 75%) and lepromatous ($N = 11$; 69%) patients. There was a significantly increased (2×) amount of Tregs (CD25+ Foxp3+) infiltrating the cutaneous lesions in lepromatous patients compared with those Tregs from tuberculoid patients (Figures 2A and 3). In lepromatous patients, Tregs were intermingled with vacuolized hysticote infiltrates all over the lesion, whereas in tuberculoid patients, rare Tregs were seen, usually within the granuloma (Figure 3). No labeled cells were seen outside the inflamed areas. Tregs were rarely found in normal human skin; of 10 normal skin biopsies, no immunostained cells were identified in 8 biopsies, whereas in 2 biopsies, only one immunostained cell was counted in all fields. Analysis of the expression of IL-10 and TGF-β showed that IL-10 but not TGF-β was significantly more expressed in lesions of lepromatous than tuberculoid patients (Figure 2B and D). CTLA-4 expression was also significantly higher in lepromatous than tuberculoid lesions (Figure 2C).

DISCUSSION

Antigen-specific T-cell anergy is a major feature of the severe forms of chronic granulomatous infectious diseases. The mechanisms driving this anergy are still a matter of debate. Elucidation of the mechanisms is an important issue, because it may facilitate the development of new strategies to treat these often serious infectious challenges. This issue has been renewed with the characterization of a subset of T cells with regulatory properties (Tregs)[4] that have the capacity to disarm effector T cells and thus, prevent disease-associated pathology.[5] However, in the case of infectious diseases, this prevention may be at the cost of reduced effectiveness of the immune response against the pathogen.[4,15] In fact, Tregs have been shown to contribute to the failure of the host in control-ling the invading organisms, representing a mechanism that leads to persistence of the pathogen and consequently, chronic inflammation.[16] Although better characterized in experimental models of infectious diseases, Tregs data on humans are less clear. Participation of these cells in the immunopathology varies depending on the agent, localization of the disease, and clinical presentation.

In cutaneous leishmaniasis, Tregs with immunosuppressive function have been shown to accumulate in the early phase of acute lesions but decrease thereafter, and they reappear in chronic lesions.[17] They were nevertheless diminished in
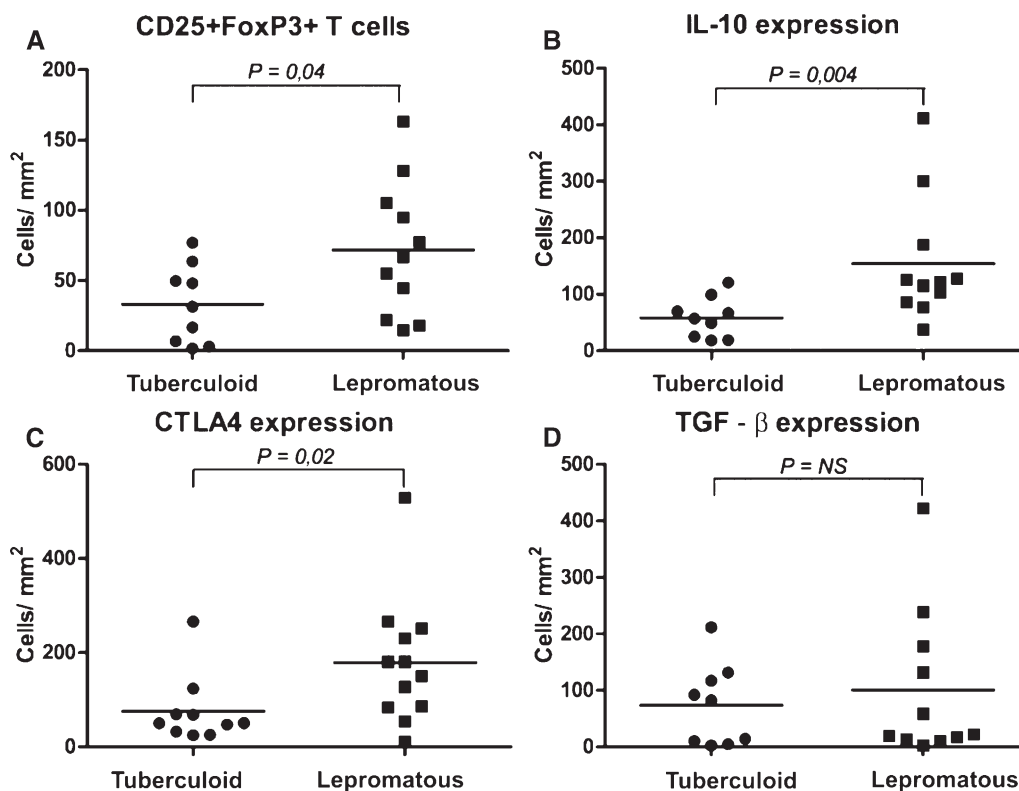
FIGURE 2. Frequency of CD25+ Foxp3+ (**A**), IL-10+ (**B**), CTLA-4+ (**C**), and TGF-β+ (**D**) cells in biopsies of lesions from tuberculoid and lepromatous leprosy patients. Each dot represents a sample; horizontal lines represent mean values.

peripheral blood compared with healthy subjects.[17] Other works also did not find increased numbers of Tregs in peripheral blood of chronic cutaneous leishmaniasis, although these cells overexpressed the inhibitory molecule CTLA-4.[10] They, however, accumulated in skin lesions and showed immunosuppressive activity. Paradoxically, Tregs seem not to play a role in visceral leishmaniasis, and they are not expanded or accumulated in the peripheral blood or spleen of these patients.[18] On the contrary, they were found to play a role in post–kala-azar dermal leishmaniasis patients in India[19] but not Sudanese patients.[20] Similarly, Tregs with suppressive functions were increased in the peripheral blood of tuberculosis patients with disseminated disease but not in those patients with localized pulmonary disease.[9] They also accumulated in disease sites relative to peripheral blood. Part of this variability may eventually relate to different methodologies used to characterize Tregs, which in most studies based on the expression of CD25 or Foxp3 but not in the coexpression of both molecules.

However, the role of Tregs in leprosy has been addressed in only a few studies, and a clear picture has not emerged.[21,22] We show here that Tregs are present in increased numbers and may have a pathogenic role in leprosy patients harboring uncontrolled bacillary multiplication (lepromatous leprosy) but not in those individuals capable of limiting *M. leprae* growth (tuberculoid leprosy). Increased frequency of CD25+ FoxP3+ T cells was seen both in *in vitro M. leprae*-stimulated PBMCs and disease sites. Our results differ from those results of another group, which found higher frequency of circulating Tregs in the peripheral blood of tuberculoid patients than lepromatous patients.[21] Diversity in cytometry strategy for defining the Treg population may account, at least in part, for the differences. In addition, this work studied *ex vivo* cells, whereas we

analyzed *in vitro*-stimulated cells. Thus, altered Treg numbers among *ex vivo* PBMCs may reflect mainly the redistribution of these cells from peripheral blood to disease sites. FoxP3+ Tregs have also been previously detected in biopsies of leprosy patients.[22] However, no statistical differences between Tregs frequency in the distinct categories of patients could be detected in that study, probably because of the limited number of patients within each category of the leprosy spectrum. Moreover, neither *in situ* double staining (CD25+/FoxP3+) nor appropriate quantitative imaging analysis could be performed in this study.[22]

We also showed that the increased expression of Tregs was paralleled by higher *in situ* expression of CTLA-4 and IL-10 but not TGF-β, suggesting potential mechanisms by which these cells could suppress CD4+ T cell responses. Although several studies verified the *in vitro* immunosuppressive ability of Tregs isolated from sites of disease, the mechanisms through which Tregs exerted this ability remained to be determined. Expression of both TGF-β and IL-10 was reported to be augmented in tuberculosis patients, but this expression was associated with other cells than Tregs.[9] In cutaneous leishmaniasis, a potential role was attributed to IL-10, with levels that were correlated with the parasite burden,[17] whereas others showed increased release of both IL-10 and TGF-β by Tregs derived from skin lesions as well as increased CTLA-4 expression by these cells.[10] Increased IL-10 expression but not CTLA-4 expression was associated with increased parasite burden in post–kala-azar dermal leishmaniasis.[19] A limitation of our study is the lack of functional analysis of Tregs and related immunoregulatory molecules to provide direct evidence of their participation in the immunopathology of the polar forms of leprosy. However, Tregs do not exert their immunosuppressive action systemically but rather *in situ*
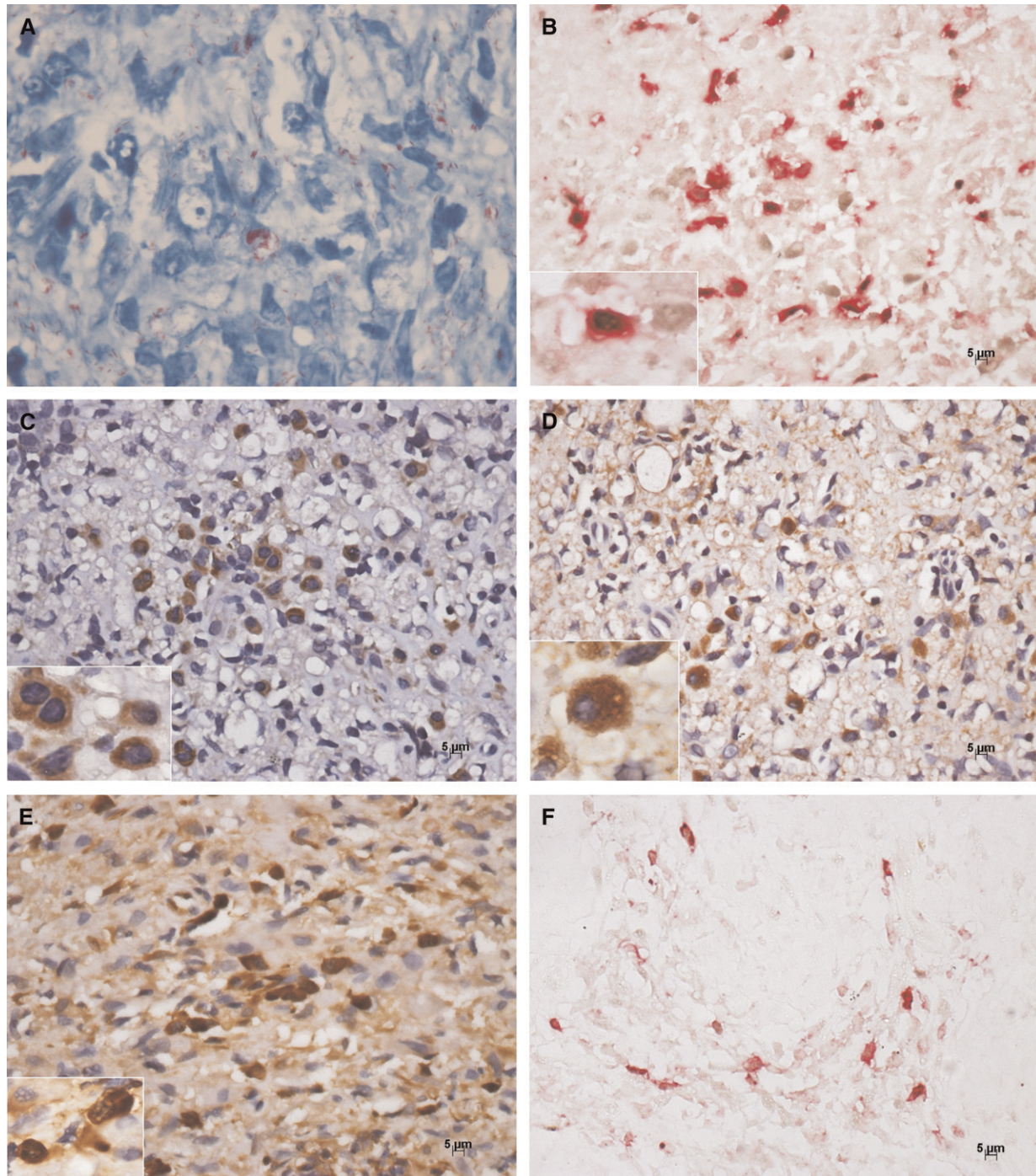
FIGURE 3. Representative sections of (**A**) Fite–Faraco staining showing numerous bacilli in purple (lepromatous patient) and (**B–F**) IHC staining for (**B**) CD25 (cytoplasmatic) and Foxp3 (nuclear), (**C**) IL-10, (**D**) CTLA-4, and (**E**) TGF-β in lepromatous leprosy and (**F**) CD25 and Foxp3 in tuberculoid leprosy. Note the high number of double-positive (CD25/Foxp3) cells intermingled with the vacuolized hysticyte infiltrate all over the lesion in **B**, whereas in **F**, rare double-positive cells were seen within the granuloma. Original magnification: **A**, 1,000×; **B–F**, 400×. Insets show the intracellular staining in more detail.

(i.e., at sites of pathogen replication).[15] The importance of *in situ* findings is illustrated by studies of chronic HIV-infected patients showing that, although the frequency of peripheral blood Tregs was not elevated, their expression in the gut was highly increased and correlated with the patients' HIV replicative status.[23] Thus, our findings of increased expression of Tregs and IL-10 not only in blood but especially *in situ*, together with the increased *in situ* expression of CTLA-4, do

suggest a suppressive role for Tregs in lepromatous patients. The increased Tregs expression could mediate the depressed antigen-specific proliferative responses and reduced IFN-γ release observed in our lepromatous patients, resulting in deficient Th-1 responses and lack of control of the bacillary load.

In fact, our observation of high number of *in situ* Tregs in lepromatous leprosy may help to explain earlier findings of reduced numbers of IL-2–producing cells in lepromatous

biopsies compared with tuberculoid biopsies.[24] A role for suppressor (regulatory) cells was also suggested in sequential IHC studies of lepromin reactions by the same group.[25] Although the presence of a high number of IL-2–producing cells was equally observed in the early phase of lepromin reaction of lepromatous and tuberculoid patients, at later phases these cells persisted in tuberculoid patients but markedly decreased in lepromatous patients.

In conclusion, as in other chronic granulomatous diseases, our results suggest that Tregs may play a role in the outcome of the host–parasite interaction in leprosy. Providing a balanced level of Treg function to achieve an appropriate level of parasite control without inducing immunopathology would be a major goal in the management of this still-challenging infectious disease.

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