Development of Type 2, But Not Type 1, Leprosy Reactions is Associated with a Severe Reduction of Circulating and In situ Regulatory T-Cells

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Abstract. Leprosy is frequently complicated by the appearance of reactions that are difficult to treat and are the main cause of sequelae. We speculated that disturbances in regulatory T-cells (Tregs) could play a role in leprosy reactions. We determined the frequency of circulating Tregs in patients with type 1 reaction (T1R) and type 2 reaction (T2R). The in situ frequency of Tregs and interleukin (IL)-17, IL-6, and transforming growth factor beta (TGF)- β -expressing cells was also determined. T2R patients showed markedly lower number of circulating and in situ Tregs than T1R patients and controls. This decrease was paralleled by increased in situ IL-17 expression but decreased TGF- β expression. Biopsies from T1R and T2R patients before the reaction episodes showed similar number of forkhead box protein P3 + (FoxP3+) and IL-17+ cells. However, in biopsies taken during the reaction, T2R patients showed a decrease in Tregs and increase in IL-17+ cells, whereas T1R patients showed the opposite: Tregs increased but IL-17+ cells decreased. We also found decreased expansion of Tregs upon in vitro stimulation with *Mycobacterium leprae* and a trend for lower expression of FoxP3 and the immunosuppressive molecule cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) in T2R Tregs. Our results provide some evidence to the hypothesis that, in T2R, downmodulation of Tregs may favor the development of T-helper-17 responses that characterize this reaction.

INTRODUCTION

Leprosy is a treatable infectious disease that is frequently complicated by the sudden appearance of reaction episodes. This is a major concern because: 1) leprosy sequelae such as nerve function impairment and disabilities are in most cases linked to these episodes and 2) treatment of these episodes is based on prolonged use of drugs with potential severe side effects, such as corticosteroids and thalidomide.¹ These episodes represent an exacerbation of the inflammatory responses and can appear before, during, or after chemotherapy for *Mycobacterium leprae*. Two types of reactions are recognized, with distinct clinical and histopathological features. Type 1 reaction (T1R) or reversal reaction is estimated to result from exacerbation of established or incipient T-helper 1 responses, presumably triggered by increased release of mycobacteria antigens in immunologically instable forms of the disease (borderline tuberculoid [BT], borderline borderline [BB], and borderline lepromatous [BL]).² Type 2 reaction (T2R) or erythema nodosum leprosum affects patients with the BB, BL, and lepromatous leprosy (LL) forms and has been defined as an immunocomplex-mediated disease. More recent evidence suggests that it is a more complex phenomenon with involvement of cell-mediated immunity of both the T-helper (Th)-1 and Th-2 phenotypes.³ However, accumulated data show that both pro- and anti-inflammatory cytokines participate in both types of reactions, leading to the conclusion that a simple model of Th-1 response in T1R and Th-2 response in T2R is not sufficient. This may explain why the search for immunological mediators, especially cytokines that could serve as surrogate markers for T1R and T2R, yielded inconsistent results.⁴

Unfortunately the factors that initiate the development of leprosy are still completely unknown⁵; for this reason, the risk

factors of T1R and T2R remain an open issue. Hormonal and nutritional factors appear to have an influence.^{6,7} However, a recent study of nearly 2,000 patients could not detect the influence of nutritional status and menstrual cycle on the development of reactions.⁵ In addition, this study showed that reactions are not necessarily the result of treatment and they also affect children, although increasing age was a risk factor. Studies on the polymorphisms of immune response genes showed that polymorphisms in Toll-like receptors 1 and 2 genes were associated with, respectively, lower and higher risk of developing T1R, whereas polymorphisms in tumor necrosis factor, mannose-binding lectin, and vitamin D receptor genes failed to show strong associations.^{8–10} These authors also showed that polymorphisms in the nucleotide-binding oligomerization domain containing 2 gene, which codifies a cytosolic receptor involved in detection of intracellular pathogens, were either associated with protection from T1R or associated with increased susceptibility to T2R.¹¹

We have recently witnessed a remarkable progress in the understanding of the biology of regulatory T-cells (Tregs) and an increasing recognition of their pivotal role in the regulation of immune responses in both pathological and physiological conditions. Tregs are a T-cell subpopulation specialized for immune suppression and engaged in the maintenance of immunological self-tolerance and homeostasis.¹² Although no single marker uniquely identifies this suppressor subpopulation, Tregs are defined as CD25 (interleukin-2 [IL-2] receptor α -chain)-expressing CD4+ T cells, which express the transcription factor forkhead box protein P3 (FoxP3), a master regulator for the development and function of Tregs.¹³ We and others have previously shown that Tregs are augmented in the anergic forms of leprosy (BL/LL) as compared with the tuberculoid leprosy/BT forms.^{14–17} Thus, Tregs seem to play an important role in the regulation of the patients' cellular immune responses to *M. leprae*, particularly in the anergy of LL.¹⁸

Although T1R and T2R are perceived as being triggered by different, but not yet well-defined, immunopathogenic

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mechanisms,¹ it is clear that they both result from exacerbation of an ongoing immune response and, as such, from lack of immune regulation. However, the participation of Tregs in the development of leprosy reaction episodes remains unclear. We then speculated that disturbances in Tregs could play a role in leprosy reactions, especially T1R, considered to result from exacerbated cell-mediated immunity. Here we show that, unexpectedly, development of T2R, instead of T1R, is associated with a sharp reduction in both in situ and circulating Tregs.

PATIENTS AND METHODS

Patients and controls. All patients in this study were recruited from the leprosy outpatient unit of the Clinics Hospital, São Paulo, Brazil. The study population comprised 28 clinically active, severe reaction (≥ 10 lesions and, in the case of T2R, with systemic manifestations and severe pain) leprosy patients who were graded clinically and histologically on the leprosy spectrum according to the Ridley–Jopling classification.¹⁹ Diagnosis of T1R and T2R was made by two expert clinicians (MABT and JA): a total of 14 patients were in T1R and had a diagnosis of BT or BB, whereas 14 were in T2R (erythema nodosum leprosum) and had a diagnosis of BL or LL. All except three were multibacillary (MB) at the moment of diagnosis of leprosy; all received leprosy multidrug chemotherapy (MDT) according to the World Health Organization (WHO) recommendations and underwent blood collection immediately before (re)initiation of the immunosuppressive treatment (corticosteroids 0.5–1.0 mg/kg and/or thalidomide up to 300 mg/day). Some patients have been prescribed the immunosuppressive treatment of the reaction but had stopped it for > 15 days and returned because of a new severe episode of reaction, when they were included in the study. For the ex vivo, circulating Tregs measurements, three distinct groups of healthy donors were recruited: exposed individuals selected among persons living in close contact with MB leprosy patients and with in vitro T-cell reactivity to *M. leprae* as previously described¹⁴ (contacts, $N = 11$), healthy nonexposed individuals (HNE, $N = 8$), and presently healthy individuals who had previously been followed at our unit for BL/LL and recurrent episodes of severe T2R, treated with WHO MDT plus corticosteroids/thalidomide, and were considered clinically cured for at least 5 years (C-T2R, $N = 7$). For the in situ Tregs measurements, the control group consisted of biopsies taken from an active lesion of patients with MB nonreactional leprosy for diagnostic purpose (MB, $N = 12$). We adopted the following exclusion criteria: pregnancy, autoimmune diseases, use of immunosuppressive drugs for other causes than leprosy, infectious comorbidities, and age under 18 years. The study was approved by the Ethics Committee of the Clinics Hospital (protocol no. 107.460). Informed written consent was obtained from all participants. A summary of the clinical and demographic data of all participants are depicted in Supplemental Table 1.

Peripheral blood mononuclear cells isolation and culture. Peripheral blood mononuclear cell (PBMC) cultures were carried out as previously described.¹⁴ PBMCs were isolated from heparinized peripheral blood by density gradient and resuspended in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% human AB serum (Sigma-Aldrich, St. Louis, MO). PBMCs (2.5×10^6 /well) were cultivated for 96 hours in 24-well flat-bottomed plates with medium

only, a cell wall preparation of *M. leprae* (MLCwA; 4 μ g/mL), and as positive control, with phytohemagglutinin (PHA) added for the last 48 hours (5 μ g/mL; Sigma-Aldrich) at 37°C and 5% CO₂. All circulating Tregs measurements were carried out with freshly isolated cells since preliminary experiments with frozen cells showed a significant reduction in the number of Tregs.

Flow cytometry analysis of Tregs. To assess the frequency of circulating Tregs and to analyze the expression of suppressor molecules by these Tregs, ex vivo PBMCs and cells harvested from the 96-hour PBMC cultures were incubated at 4°C for 30 minutes with the following specific antibodies: anti-CD3 BV605 (clone SK7), anti-CD4 V500 (clone RPA-T4), anti-CD25 FITC (clone M-A251), anti-CD127 PE-Cy7 (clone HIL-7R-M21), anti-CD152 (CTLA-4) BV421 (clone BNI3), anti-LAP PE-CF594 (TW4-2F8), and anti-CD39 APC (clone TU66) (all from BD Biosciences, San Diego, CA). After incubation, cells were washed with phosphate-buffered saline (PBS)/bovine serum albumin (BSA). For the FoxP3 intracellular staining, cells were resuspended in Fix/Perm buffer (eBioscience, San Diego, CA) and left at 4°C for 30 minutes. Subsequently, these cells were washed with PBS/BSA and a permeabilization buffer (eBioscience) and incubated for 15 minutes with normal rat serum for preventing nonspecific staining. Next, anti-FoxP3 antibody (clone PCH101, eBioscience) was added and the cells were incubated for 30 minutes at 4°C. Cells were then washed and immediately acquired and analyzed using LSRFortessa flow cytometer (BD Biosciences, San Jose, CA). Tregs were characterized as CD4⁺ CD25⁺ CD127^{low}/FoxP3⁺ cells according to the optimized multicolor immunofluorescence panel for in-depth characterization of human Tregs (OMIP-004).²⁰ All analyses were performed in CD4⁺ cells gated within CD3⁺ cells. Illustrative examples of the gating strategy are shown in Figure 1A. CD8⁺ Tregs were searched in the CD3⁺ CD4[−] lymphocytes gate. Compensation of the subpopulations analyzed was carried out for every experiment using unstained cells and the fluorescence minus one protocol as recommended. At least 700,000 events were acquired for each analysis. Data were processed using the FlowJo 7.6.5 software (Tree Star, Inc., Ashland, OR).

Immunohistochemistry of lesion biopsies. Biopsies were taken with a standard dermatologic biopsy punch. Biopsies were preferably taken from well-circumscribed plaques in T1R and T2R patients, and in MB patients without reaction from the most infiltrated lesions. For the immunohistochemical (IHC) study of patients' biopsies, a streptavidin–biotin peroxidase method was used as previously described.¹⁴ Briefly, after deparaffinization in xylene and hydration in ethyl alcohol, endogenous peroxidase was blocked by incubating in 3% hydrogen peroxidase solution in dark chamber. Antigen recovery was performed in a retrieval solution at pH 9.0 (S2368; Dako, Carpinteria, CA) for 20 minutes at 95°C. Nonspecific proteins were blocked by incubating sections in skim milk. The primary antibodies anti-transforming growth factor beta (TGF- β) (Novocastra Leica Biosystems, New Castle, United Kingdom), anti-IL-17 (AF-317-NA; R&D Systems, Minneapolis, MN), anti-IL-6 (NCL-L-IL-6; Novocastra, Buffalo Grove, IL), and anti-FoxP3 (14-4776; eBioscience) as well as a labeled streptavidin–biotin complex (Dako, Carpinteria, CA) were applied. 3,3-Diaminobenzidine tetrahydrochloride (Sigma-Aldrich) was used as chromogen, and the slides were counterstained with hematoxylin and hydrated in alcohol. All reactions

FIGURE 1. Flow cytometry analysis of regulatory T-cells (Tregs) (CD4⁺ CD25⁺ CD127^{low/-} forkhead box protein P3 + [FoxP3⁺] cells) in peripheral blood mononuclear cells (PBMCs) of leprosy patients and controls. (A) The gate strategy showing in the upper row is the percentage of CD25⁺ cells within CD4⁺ cells and in the bottom row is the expression of FoxP3⁺ and CD127^{low/-} cells among the CD25⁺ cells. (B) Peripheral blood Treg frequency in type 1 reaction (T1R, $N = 14$), type 2 reaction (T2R, $N = 14$) patients; C-T2R ($N = 7$), contacts ($N = 10$), and healthy nonexposed individuals controls ($N = 8$). (C) Tregs frequency in PBMCs stimulated in vitro with cell wall preparation of *Mycobacterium leprae* (MLCwA) or phytohemagglutinin (PHA), or not stimulated (medium), from T1R ($N = 13$) and T2R ($N = 14$) patients and contacts ($N = 10$). Results are presented as mean $\pm$ standard error of the mean. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

were performed with positive and negative controls. The latter comprised isotype controls and omission of the primary antibody. The images were captured using AxioVision 4.8.2 software (Zeiss, Oberkochen, Germany). Six images from each specimen were considered. The area of the granulomatous inflammatory infiltrate was measured, and the stained cells were counted using Image-Pro Plus, version 6.0 (Media Cybernetics, Rockville, MD).

Statistical analysis. Analysis of variance with Newman-Keuls posttest was used for parametric data from the T1R, T2R, and control groups, whereas Kruskal-Wallis test with Dunn's posttest was used to compare the nonparametric data. Significance was set at $P < 0.05$. GraphPad-Prisma 5.0 (GraphPad Software, Inc., San Diego, CA) was used.

RESULTS AND DISCUSSION

We determined the frequency of peripheral blood Tregs during a clinically active severe T1R or T2R episode, immediately before initiation of the immunosuppressive treatment. As shown in Figure 1B, the percentage of Tregs in T2R patients was markedly reduced as compared with healthy controls, exposed or unexposed to *M. leprae* (means $\pm$ standard error: T2R, 1.7 ± 0.2 ; contacts, 3.4 ± 0.2 ; and HNE, $3.2 \pm 0.4\%$, $P < 0.001$). This percentage was also much lower than that observed in a previous study with BL/LL patients ($5.5\% \pm 0.9\%$).¹⁴ To verify whether this reduction was specifically related to the T2R episode or a constitutive deficiency

of these individuals, Tregs frequency was also determined in healthy subjects with a past history of LL and recurrent episodes of severe T2R (C-T2R). These subjects exhibited high number of circulating Tregs ($3.9\% \pm 0.5\%$, $P < 0.001$ versus T2R) (Figure 1B). On the other hand, the percentage of Tregs in T1R patients ($3.4\% \pm 0.4\%$) was similar to that from the control groups and significantly higher than that of T2R patients (Figure 1B). We then examined the possibility that the decrease in CD4⁺ Tregs would have been compensated by an increase in CD8⁺ Tregs. CD8⁺ Tregs have recently been shown to be augmented after in vitro stimulation in children with MB leprosy compared with age-matched contacts.²¹ As illustrated in Figure 1A, the presence of CD25⁺ or CD25^{high} cells within the CD8 subset (the TCD3⁺ CD4⁺ subpopulation on the left of the histograms displayed on the upper line) was negligible. The same held true for the expression of FoxP3, which was almost exclusively expressed by CD3⁺ CD4⁺ cells (not shown). Thus in our leprosy patients, CD8⁺ Tregs seem not to play a role. Next, we tested the hypothesis that the reduction in peripheral blood of Tregs was due to redistribution to the sites of leprosy reaction lesions by determining the frequency of Tregs in biopsies taken from these lesions during the same reaction episode. We found a severe reduction in Tregs frequency in T2R lesions ($P < 0.001$), whereas in T1R lesions, their frequency was comparable to the frequency found in lesions of MB patients without reaction (Figure 2A). Thus, during T2R Tregs are decreased in both peripheral blood and in situ.

FIGURE 2. Representative sections of immunofluorescence staining (upper row, original magnification 400 $\times$) and respective frequencies (bottom row) of (A) forkhead box protein P3+ (FoxP3+), (B) transforming growth factor beta (TGF- β)+, (C) interleukin (IL)-6+, and (D) IL-17+ cells in biopsies of lesions from type 1 reaction (T1R) ($N = 8$ –12), type 2 reaction (T2R) ($N = 12$ –14), and multibacillary patients ($N = 10$ –12). Results are represented as mean $\pm$ standard error of the mean. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Insets show the intracellular staining in more detail.

To understand the mechanisms underlying the Tregs decrease of T2R patients, first we determined whether these cells would have a reduced capacity to expand. This rationale was based on observations showing that Tregs accumulate within inflamed cutaneous lesions elicited by microbial challenge (e.g., mycobacteria, varicella zoster virus) and that the Tregs accumulation was associated with control of the inflammatory process.^{22,23} It was also demonstrated that the accumulation was due to local proliferation of Tregs. In fact, Tregs from leprosy reaction patients and controls were able to proliferate in vitro (Figure 1C). Interestingly however, it can be seen that although the Tregs pool of T2R patients expanded significantly, the yields induced with MLCwA were significantly lower than those resulting from MLCwA-induced expansion of Tregs from T1R patients and healthy controls; of note, in the latter two groups, the yields were comparable. On the other hand, the positive control PHA induced similar vigorous Tregs expansion in the three groups. To exclude the possibility that the reduced antigen-specific induction of Tregs proliferation was due to the lower starting input of Tregs, the percentage of Tregs in presence of medium alone was subtracted from the percentages induced with MLCwA and PHA; this analysis showed that there were still lower Tregs yields with MLCwA stimulation in T2R than in T1R and healthy controls ($P = 0.024$, Kruskal–Wallis test), but not with PHA ($P > 0.05$). Our results point to a reduction in the in vitro *M. leprae*-induced replicative capacity of T2R Tregs but not of T1R Tregs.

Second, we determined the in situ expression of IL-6, IL-17, and TGF- β . Although TGF- β is a prerequisite for differentiation of both Th-17 and Tregs, IL-6 turns off the pathway to Tregs reprogramming the differentiation toward Th-17 cells.^{24,25} We then expected that the differences in in situ Tregs numbers would reflect the balance between these cytokines in the inflammatory microenvironment of T1R and T2R. Surprisingly, we found that T2R lesions exhibited a marked reduction in the frequency of TGF- β + cells as compared with T1R and MB lesions, which in turn expressed similar high levels (Figure 2B). On the other hand, while the number of IL-6-

expressing cells was low in T2R, T1R, and MB lesions (all means ≤ 32 cells/mm²; Figure 2C), IL-17+ cell results mirrored those of FoxP3 and TGF- β : there was a high expression in T2R (mean 295 ± 49 cells/mm²) and a significantly lower expression in T1R (114 ± 27 cells/mm²) and MB lesions (136 ± 22 cells/mm²) (Figure 2D). The low IL-6 expression was somewhat disappointing because there is evidence for: 1) elevated IL-6 levels in the serum of T2R patients and 2) an association between some IL-6 gene single-nucleotide polymorphisms and T2R.^{26,27} However, elevated IL-6 serum levels have also been described in BL/LL patients without reaction as well as in T1R patients.^{28–30} In addition, IL-6 messenger RNA and protein have been detected in T1R lesions.^{31–33} Thus, the lack of unequivocal correlations between specific cytokines and distinct phenotypes or outcomes highlights the complexity of the immune response in leprosy. An exception to this could be the high expression of IL-17, which is in agreement with the neutrophil infiltration and Th-17 pattern of immune response that characterize the histological picture of T2R.³⁴ Although the underlying mechanisms remain unclear, our data may suggest that in T2R skin lesions, Tregs development is turned off to make room for the proinflammatory Th-17-mediated responses.

We additionally tested the possibility that IL-6 played an inhibitory role on the in vitro Tregs expansion induced with MLCwA and PHA. Concentrations ranging from 10 to 200 pg/mL of IL-6 were unable to block the expansion of Tregs induced with MLCwA and PHA in PBMC of reaction patients and contacts (data not shown). In fact, the role played by IL-6 in the inhibition of Tregs has been debated and it has been suggested that this cytokine preferentially drives the differentiation of Th-0 cells toward Th-17 cells through the blockade of the pathway to Tregs, but does not directly inhibit the proliferation of already differentiated Tregs.^{35,36}

We were able to retrieve the biopsy taken at the moment of diagnosis of leprosy in five T1R and eight T2R patients, before they developed the reaction episodes. We then compared these biopsies with those taken during the reactions.

FIGURE 3. Frequency of (A) forkhead box protein P3+ (FoxP3+), (B) interleukin (IL)-17 +, and (C) IL-6 + cells in biopsies of lesions from five type 1 reaction (T1R) and eight type 2 reaction (T2R) patients before (BR) and during reaction episodes. Results are presented as mean $\pm$ standard error of the mean. ** $P < 0.01$.

As shown in Figure 3A, before reaction, the frequency of Tregs from the patients who subsequently developed T1R and T2R were similar ($P = 0.7$). However, during the reaction episodes, the frequency increased 2.5 times in T1R lesions and decreased by 60% in T2R lesions, resulting in a frequency of Tregs in T2R lesions that was 4-fold lower than in T1R lesions ($P < 0.01$). Thus, development of T2R, but not T1R, was associated with a sharp decrease of in situ Tregs. We also compared these biopsies with regard to IL-17 and IL-6 expression (Figure 3B and 3C). Interestingly, IL-17 expression was also comparable before reaction, but during T2R, the frequency of IL-17+ cells increased while in T1R it decreased, resulting in a frequency in T2R lesions 5-fold higher than in T1R lesions ($P < 0.01$). IL-6 expression was low before and remained unchanged during both T1R and T2R. These data corroborate the notion that development of T2R is accompanied by a sharp decrease in Tregs and an increase in IL-17 expression.

Finally, although this study was not designed to examine the functional properties of Tregs, we determined the levels of expression of some molecules known to mediate the immunosuppressive activity of Tregs, and thus tried to infer indirectly the immunosuppressive potential of these cells. We measured the surface expression of 1) CTLA-4, a molecule that competes with CD28 for binding to CD80/CD86 costimulatory molecules, downmodulating T-cell receptor signaling and IL-2 secretion³⁷; 2) CD39, a molecule that hydrolyzes the extracellular adeno-

sine triphosphate or adenosine diphosphate released by T-cells during antigen presentation to adenosine monophosphate, displaying inhibitory and antiproliferative effects³⁸; and 3) latency-associated peptide (LAP), which is a TGF- β -associated molecule that acts as a transporter of latent TGF- β to the cell membrane and then releases the cytokine to the extracellular milieu.³⁹ We additionally assessed the median fluorescence intensity of FoxP3, whose intracellular accumulation correlates with the immunosuppressive capacity of the Tregs.⁴⁰ The ex vivo expression levels of these molecules by Tregs are shown in Figure 4. As in previous studies,⁴¹ there was individual variability in the expression of these molecules in the three groups and no significant differences among them were found, although T2R patients showed a trend for lower expression of CTLA-4 and FoxP3. We also assessed the in vitro expression of these molecules in unstimulated and MLCwA and PHA-stimulated Tregs (Table 1). Similarly, while no significant differences could be detected among the groups, a trend for lower expression of CTLA-4 in the three in vitro conditions and for lower FoxP3 median fluorescence intensity in MLCwA cultures was noted in Tregs from T2R patients. Thus, although this limited set of experiments did not allow for definite conclusions, our data suggest that, in addition to their reduced frequency, T2R Tregs may present some functional impairment.

CONCLUSION

We show that T2R develops in leprosy patients concomitantly with a reduction in the Tregs frequency in peripheral blood and in situ, while such association is not seen in T1R. Although the two types of reaction are clinically and histologically distinct, the attempts to define a distinct pattern of immunological markers, which would underlie each type of reaction were hindered because these markers frequently overlap. Recently, a large IHC study of leprosy biopsies reported that in situ TGF- β expression was associated with T1R, although this association showed low specificity as high TGF- β expression was also found in 50% of the LL biopsies and 54% of the T2R biopsies.⁴² We verified high and comparable expression of TGF- β in MB and T1R biopsies, while its expression was at least 50% lower in T2R. We also assessed the in situ expression of IL-17 and IL-6 and noted high IL-17 expression in T2R but low expression in T1R and MB lesions

FIGURE 4. Percentage of regulatory T-cells (Tregs) expressing surface molecules (CTLA-4, latency-associated peptide [LAP], and CD39) and forkhead box protein P3 (FoxP3) median fluorescence intensity (MFI) by Tregs from type 1 reaction (T1R) ($N = 8-10$) and type 2 reaction (T2R) ($N = 11-13$) patients and contacts ($N = 7-10$). Box plots show the median and interquartiles (25-75%).

TABLE 1
Percentage of Tregs expressing surface molecules and FoxP3 MFI in PBMCs stimulated culture from T1R and T2R and contacts

	T1R	T2R	Contacts
CTLA-4	N = 10	N = 8	N = 8
Medium	9.17% (5.46–21.55)	5.21% (5.10–11.7)	12.9% (5.84–19.05)
MLCwA	16.50% (5.91–26.50)	7.82% (5.57–16.90)	18.8% (7.29–27.78)
PHA	78.85% (49.78–88.43)	59.05% (53.20–69.15)	80.35% (62.08–87.78)
LAP	N = 9	N = 7	N = 8
Medium	3.60% (2.21–11.73)	5.56% (4.22–13.7)	3.19% (1.61–4.72)
MLCwA	5.50% (3.18–9.29)	3.14% (1.42–10.28)	3.64% (2.14–6.73)
PHA	12.10% (7.28–19.30)	14.80% (11.80–20.05)	13.35% (9.84–19.50)
CD39	N = 9	N = 7	N = 10
Medium	52.70% (22.20–78.60)	50.75% (15.83–73.95)	70.70% (58.18–76.20)
MLCwA	25.10% (13.25–61.30)	39.35% (12.07–55.68)	42.75% (38.75–59.50)
PHA	18.25% (12.7–40.85)	18.40% (13.10–26.50)	16.50% (13.28–17.40)
FoxP3 (MFI)	N = 13	N = 12	N = 10
Medium	1,440 (773.2–2,802)	1,576 (926.8–3,979)	1,032 (709.5–2,274)
MLCwA	2,818 (1,435–3,520)	1,846 (1,002–2,374)	2,567 (1,572–3,707)
PHA	2,749 (1,788–4,592)	4,972 (2,330–14,915)	3,691 (2,774–11,562)

FoxP3 = forkhead box protein P3; LAP = latency-associated peptide; MFI = median fluorescence intensity; MLCwA = cell wall preparation of *Mycobacterium leprae*; PBMCs = peripheral blood mononuclear cells; PHA = phytohemagglutinin; Tregs = regulatory T-cells; T1R = type 1 reaction; T2R = type 2 reaction.
Median and interquartile interval (25–75%).

while IL-6 expression was uniformly low in the three groups. Thus, our expectation that high TGF- β and IL-6 expression would explain the downmodulation of Tregs by one side and the upregulation of Th-17 type response by the other side was not confirmed. Other regulatory cytokines such as IL-1 β , IL-21, IL-22, and IL-23 should be investigated, among others, that could explain the immune regulation that takes place in T2R. In T1R, the mechanisms leading to lack of control of the exacerbated responses remain to be determined.

In addition, our assays on Tregs capacity to expand and on the expression of molecules associated with immunosuppressive activity suggest that T2R Tregs may also present some dysfunction. Overall, our results provide some evidence to the hypothesis that, in T2R, downmodulation of Tregs would favor the development of Th-17 responses that characterize this type of reaction. We thus believe that better understanding of the role played by Tregs in reaction episodes can possibly provide a new target for the treatment of this still-challenging complication of leprosy.

Received September 14, 2015. Accepted for publication December 28, 2015.

Published online February 22, 2016.

Note: Supplemental table appears at www.ajtmh.org.

Acknowledgments: We thank the Mycobacteria Research Laboratories, Colorado State University, for kind donation of the *Mycobacterium leprae* antigen; Naiura Vieira Pereira and Luciane Kanashiro Gallo for the assistance with the immunohistochemistry; and Luiz Augusto M. Fonseca for careful English editing.

Financial support: This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo no. 2014/15286-0, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, and Fundação Paulista contra a Hanseníase. GB and AJSD are senior researchers of Conselho Nacional de Desenvolvimento Científico e Tecnológico.

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REFERENCES

- Kahawita IP, Walker SL, Lockwood DL, 2008. Leprosy type 1 reactions and erythema nodosum leprosum. *An Bras Dermatol* 83: 75–82.
- Walker SL, Lockwood DN, 2008. Leprosy type 1 (reversal) reactions and their management. *Lepr Rev* 79: 372–386.
- Kahawita IP, Lockwood DNJ, 2008. Towards understanding the pathology of erythema nodosum leprosum. *Trans R Soc Trop Med Hyg* 102: 329–337.
- Pandhi D, Chhabra N, 2013. New insights in the pathogenesis of type 1 and type 2 lepra reaction. *Indian J Dermatol Venereol Leprol* 79: 739–749.
- Scollard DM, Martelli CMT, Stefani MMA, Maroja MF, Villahermosa L, Pardillo F, Tamang KB, 2015. Risk factors for leprosy reactions in three endemic countries. *Am J Trop Med Hyg* 92: 108–114.
- Saunderson P, Gebre S, Byass P, 2000. Reversal reactions in the skin lesions of AMFES patients: incidence and risk factors. *Lepr Rev* 71: 309–317.
- Saporta L, Yuxsel A, 1994. Androgenic status in patients with lepromatous leprosy. *Br J Urol* 74: 221–224.
- Misch EA, Macdonald M, Ranjit C, Sapkota BR, Wells RD, Siddiqui MR, Kaplan G, Hawn TR, 2008. Human TLR1 deficiency is associated with impaired mycobacterial signaling and protection from leprosy reversal reaction. *PLoS Negl Trop Dis* 2: e231.
- Bochud PY, Hawn TR, Siddiqui MR, Saunderson P, Britton S, Abraham I, Argaw AT, Janer M, Zhao LP, Kaplan G, Aderem A, 2008. Toll-like receptor 2 (TLR2) polymorphisms are associated with reversal reaction in leprosy. *J Infect Dis* 197: 253–261.
- Sapkota BR, Macdonald M, Berrington WR, Misch EA, Ranjit C, Siddiqui MR, Kaplan G, Hawn TR, 2011. Association of TNF, MBL, and VDR polymorphisms with leprosy phenotypes. *Hum Immunol* 10: 992–998.
- Berrington WR, Macdonald M, Khadge S, Sapkota BR, Janer M, Hage DA, Kaplan G, Hawn TR, 2010. Common

- polymorphisms in the *NOD2* gene region are associated with leprosy and its reactive states. *J Infect Dis* 201: 1422–1435.
12. Sakaguchi S, Yamaguchi T, Nomura T, Ono M, 2008. Regulatory T cells and immune tolerance. *Cell* 133: 775–787.
 13. Hori S, Nomura T, Sakaguchi S, 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057–1061.
 14. Palermo ML, Pagliari C, Trindade MA, Yamashitafuji TM, Duarte AJ, Cacere CR, Benard G, 2012. Increased expression of regulatory T cells and down-regulatory molecules in lepromatous leprosy. *Am J Trop Med Hyg* 86: 878–883.
 15. Saini C, Ramesh V, Nath I, 2014. Increase in TGF- β secreting CD4⁺CD25⁺ FOXP3⁺ T regulatory cells in anergic lepromatous leprosy patients. *PLoS Negl Trop Dis* 8: e2639.
 16. Kumar S, Naqvi RA, Ali R, Rani R, Khanna N, Rao DN, 2013. CD4⁺CD25⁺ T regs with acetylated FoxP3 are associated with immune suppression in human leprosy. *Mol Immunol* 56: 513–520.
 17. Quaresma JA, Esteves PC, de Sousa Aarão TL, de Sousa JR, da Silva Pinto D, Fuzii HT, 2014. Apoptotic activity and Treg cells in tissue lesions of patients with leprosy. *Microb Pathog* 76: 84–88.
 18. Bobosha K, Wilson L, van Meijgaarden KE, Bekele Y, Zewdie M, Schip JJ, Abebe M, Hussein J, Khadge S, Neupane KD, Hage DA, Jordanova ES, Aseffa A, Ottenhoff TH, Geluk A, 2014. T-cell regulation in lepromatous leprosy. *PLoS Negl Trop Dis* 8: e2773.
 19. Ridley DS, Jopling WH, 1966. Classification of leprosy according to immunity. A five-group system. *Int J Lepr Other Mycobact Dis* 34: 255–273.
 20. Biancotto A, Dagur PK, Fuchs JC, Langweiler M, McCoy JP Jr, 2012. OMIP-004: in-depth characterization of human T regulatory cells. *Cytometry A* 81: 15–16.
 21. Fernandes C, Gonçalves HS, Cabral PB, Pinto HC, Pinto MIM, Câmara LMC, 2013. Increased frequency of CD4 and CD8 regulatory T cells in individuals under 15 years with multi-bacillary leprosy. *PLoS One* 8: e79072.
 22. Vukmanovic-Stejić M, Agius E, Booth N, Dunne PJ, Lacy KE, Reed JR, Sobande TO, Kissane S, Salmon M, Rustin MH, Akbar AN, 2008. The kinetics of CD4⁺Foxp3⁺ T cell accumulation during a human cutaneous antigen-specific memory response in vivo. *J Clin Invest* 118: 3639–3650.
 23. Vukmanovic-Stejić M, Sandhu D, Sobande TO, Agius E, Lacy KE, Riddell N, Montez S, Dintwe OB, Scriba TJ, Breuer J, Nikolich-Zugich J, Ogg G, Rustin MH, Akbar AN, 2013. Varicella zoster-specific CD4⁺Foxp3⁺ T cells accumulate after cutaneous antigen challenge in humans. *J Immunol* 190: 977–986.
 24. Noack M, Miossec P, 2014. Th17 and regulatory T cell balance in autoimmune and inflammatory diseases. *Autoimmun Rev* 13: 668–667.
 25. Ouyang W, Kolls JK, Zheng Y, 2008. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity* 28: 454–467.
 26. Stefani MM, Guerra JG, Sousa AL, Costa MB, Oliveira ML, Martelli CT, Scollard DM, 2009. Potential plasma markers of type 1 and type 2 leprosy reactions: a preliminary report. *BMC Infect Dis* 27: 9–75.
 27. Sousa AL, Fava VM, Sampaio LH, Martelli CM, Costa MB, Mira MT, Stefani MM, 2012. Genetic and immunological evidence implicates interleukin 6 as a susceptibility gene for leprosy type 2 reaction. *J Infect Dis* 205: 1417–1424.
 28. Chaitanya VS, Lavania M, Nigam A, Turankar RP, Singh I, Horo I, Sengupta U, Jadhav RS, 2013. Cortisol and proinflammatory cytokine profiles in type 1 (reversal) reactions of leprosy. *Immunol Lett* 156: 159–167.
 29. Belgaumkar VA, Gokhale NR, Mahajan PM, Bharadwaj R, Pandit DP, Deshpande S, 2007. Circulating cytokine profiles in leprosy patients. *Lepr Rev* 78: 223–230.
 30. Iyer A, Hatta M, Usman R, Luiten S, Oskam L, Faber W, Geluk A, Das P, 2007. Serum levels of interferon-gamma, tumour necrosis factor-alpha, soluble interleukin-6R and soluble cell activation markers for monitoring response to treatment of leprosy reactions. *Clin Exp Immunol* 150: 210–216.
 31. Moraes MO, Sarno EN, Teles RMB, Almeida AS, Saraiva BC, Nery JAC, Sampaio EP, 2000. Anti-inflammatory drugs block cytokine mRNA accumulation in the skin and improve the clinical condition of reactional leprosy patients. *J Invest Dermatol* 115: 935–941.
 32. Atkinson SE, Khanolkar-Young S, Marlowe S, Jain S, Reddy RG, Suneetha S, Lockwood DN, 2004. Detection of IL-13, IL-10, and IL-6 in the leprosy skin lesions of patients during prednisolone treatment for type 1 (T1R) reactions. *Int J Lepr Other Mycobact Dis* 72: 27–34.
 33. Teles RM, Moraes MO, Geraldo NT, Salles AM, Sarno EN, Sampaio EP, 2002. Differential TNF-alpha mRNA regulation detected in the epidermis of leprosy patients. *Arch Dermatol Res* 294: 355–362.
 34. Martiniuk F, Giovinazzo J, Tan AU, Shahidullah R, Haslett P, Kaplan G, Levis WR, 2012. Lessons of leprosy: the emergence of TH17 cytokines during type II reactions (ENL) is teaching us about T-cell plasticity. *J Drugs Dermatol* 11: 626–630.
 35. Goodman WA, Levine AD, Massari JV, Sugiyama H, McCormick TS, Cooper KD, 2009. IL-6 signaling in psoriasis prevents immune suppression by regulatory T cells. *J Immunol* 183: 3170–3176.
 36. Fujimoto M, Nakano M, Terabe F, Kawahata H, Ohkawara T, Han Y, Ripley B, Serada S, Nishikawa T, Kimura A, Nomura S, Kishimoto T, Naka T, 2011. The influence of excessive IL-6 production in vivo on the development and function of Foxp3⁺ regulatory T cells. *J Immunol* 186: 32–40.
 37. Walunas TL, Lenschow DJ, Bakker CY, Linsley OS, Freeman GJ, Green JM, Thompson CB, Bluestone JA, 1994. CTLA-4 can function as a negative regulator of T-cell activation. *Immunity* 1: 405–413.
 38. Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R, Höpner S, Centonze D, Bernardi G, Dell'Acqua ML, Rossini PM, Battistini L, Röttschke O, Falk K, 2007. Expression of ectonucleotidase CD39 by Foxp3⁺ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood* 110: 1225–1232.
 39. Lawrence DA, 2001. Latent-TGF-beta: an overview. *Mol Cell Biochem* 219: 163–170.
 40. Chauhan SK, Saban DR, Lee HK, Dana R, 2009. Levels of Foxp3 in regulatory T cells reflect their functional status in transplantation. *J Immunol* 182: 148–153.
 41. Pulte ED, Broekman MJ, Olson KE, Drosopoulos JHF, Kizer JR, Islam N, Marcus AJ, 2007. CD39/NTPDase-1 activity and expression in normal leukocytes. *Thromb Res* 121: 309–317.
 42. Lockwood DN, Suneetha L, Sagili KD, Chaduvula MV, Mohammed I, van Brakel W, Smith WC, Nicholls P, Suneetha S, 2011. Cytokine and protein markers of leprosy reactions in skin and nerves: baseline results for the North Indian INFIR cohort. *PLoS Negl Trop Dis* 5: e1327.