T regulatory cells (TREG)(TCD4+CD25+FOXP3+) distribution in the different clinical forms of leprosy and reactional states*

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Abstract: BACKGROUND: Leprosy is characterized histologically by a spectrum of different granulomatous skin lesions, reflecting patients' immune responses to Mycobacterium leprae. Although CD4+CD25+ FoxP3+ T regulatory cells are pivotal in the immuneregulation, presence, frequency, and distribution of Tregs in leprosy, its reactional states have been investigated in few studies.
OBJECTIVES: This study aimed to verify the frequency and distribution of regulatory T cells in different clinical forms and reactional states of leprosy.
METHODS: We performed an immunohistochemical study on 96 leprosy cases [Indeterminate (I): 9 patients; tuberculoid tuberculoid: 13 patients; borderline tuberculoid: 26 patients; borderline borderline: 3 patients; borderline lepromatous: 8 patients; lepromatous lepromatous: 27 patients; reversal reaction: 8 patients; and erythema nodosum leprosum: 2 patients].
RESULTS: FoxP3-positive cells were present in 100% of the cases with an average density of 2.82% of the infiltrate. Their distribution was not related to granulomatous structures or special locations. There was a statistically significant increment of FoxP3 expression in patients with leprosy reversal reactions when compared with patients presenting with type I leprosy (P= 0.0228); borderline tuberculoid leprosy (P = 0.0351) and lepromatous leprosy (P = 0.0344).
CONCLUSIONS: These findings suggest that Tregs play a relevant role in the etiopathogenesis of leprosy, mainly in type I leprosy reaction.
Keywords: Leprosy; T-Lymphocytes; T-Lymphocytes, regulatory

INTRODUCTION

Leprosy or Hansen disease (HD) is characterized by a wide spectrum of histologically distinct granulomatous skin lesions, reflecting patients' immune responses to *Mycobacterium leprae* (*M. leprae*), varying from predominantly epithelioid cells with absence or occasional presence of bacilli at the tuberculoid end of the spectrum (TT), to abundance of bacilli-filled foamy macrophages at the lepromatous leprosy (LL) pole.[1]

Between the leprosy polar forms (TT and LL), we find the borderline forms which are immunologically unstable, and classified into intermediate subgroups defined as borderline lepromatous (BL), borderline borderline (BB), and borderline tuberculoid (BT), leprosy. [2] It is known that nearly 30% of the individuals with borderline leprosy may present type-I reaction, and erythema nodosum leprosum (ENL) is observed in up to 50% of lepromatous leprosy patients receiving antimycobacterial therapy.[3,4]

Tregs are T-lymphocytes; $CD4^+$ $CD25^+FoxP3^+$ represents up to 10% of T $CD4^+$ cells found in the peripheral blood of normal mice and human beings.[5] Characteristically, they express CD25 and the forkhead family transcription factor FoxP3, which plays a vital role in the generation of Treg $CD25^+$ high, and it is the most specific marker currently available.[6] Tregs are critically involved in balancing the reactivity of the immune system and preventing autoimmunity. Parallel to their role in preventing autoimmune reactions, Tregs have been shown to control excessive inflammatory responses against pathogens.[7]

However, a strict control of T effector cell responses by Tregs may favor infection and promote pathogen persistence.[8,9]

Tregs have been described in: infectious diseases (leishmaniasis, tuberculosis); autoimmune diseases like lupus erythematosus, psoriasis, graft-versus-host disease, sarcoidosis; and in cutaneous cancer (basal cell carcinoma, mycosis fungoides, and Sezary syndrome).[10,11]

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Recently, note was taken of a statistically significant difference in FoxP3 expression in patients with leprosy reversal reactions (BT-RR and BB-RR), when compared with the non-reaction forms of the disease (BL, BT, LL, TT)[1]

Another study reported that circulating Tregs increased in TT patients, suggesting that, rather than being detrimental to immunity, the activity of intact Tregs could be beneficial to leprosy patients.[12]

Furthermore, another recent study has demonstrated 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 individuals capable of limiting *M. leprae* growth (tuberculoid leprosy).[13]

In order to further knowledge about the role of Tregs in leprosy, we performed a retrospective study on 96 cases of HD to investigate its presence, frequency, and the distribution of Tregs in skin lesions.

MATERIALS AND METHODS

An observational, descriptive and retrospective study was performed, based on immunohistochemical analysis of sections in paraffin, obtained from biopsies of leprosy patients.

The study population consisted of 305 blades of patients who were treated at a dermatology center from January 1 to December 31, 2008, diagnosed with leprosy confirmed by histopathological examination, and whose paraffin blocks were in good condition, filed within the Department of Histopathology.

Given a 90% sensitivity for the technique to be tested, a margin of error of 5% and a confidence level of 95%, a total of 96 cases composed the study sample, according to the formula used.[14]

$n = N.z^2.p(1-p) / d_i^2.(N-1) + z^2.p(1-p)$

p = 0.90 (expected sensitivity);
N = 305 (population size);
di = 0.05 (sample error);
z = 1.96 (for 95% confidence);
n = 96 (sample size).

Thus, the biopsy specimens of 96 blocks were randomly selected (male:female ratio = 53:43; mean age: 37.51 years; median age: 37.5 years; age range: 5–79 years).

Cutaneous biopsies had been performed at the time of diagnosis in 86 cases; 8 cases of RR were biopsied 5, 4, 2, 4, 5, 3, 4 and 3 months respectively after beginning multidrug therapy. Two cases of ENL were biopsied, respectively, 12 and 10 months after initiating multidrug therapy for LL.

Biopsy specimens were fixed in 10% buffered formalin and subsequently embedded in paraffin. Sections were stained with hematoxylin-eosin for routine histopathological evaluation.

All specimens were stained with modified Fite-Faraco stain for acid fast bacilli; the bacterial index of the granuloma was assessed using the logarithmic scale, in accordance with Ridley.[15,16]

The immunohistochemichal investigation for the detection of T $CD4^+$ $CD25^+$ $FoxP3^+$ cells was performed on histological sections taken from the 5-µm-thick biopsy specimens.

The specific monoclonal antibody Anti-FoxP3 (Clone 236A/E7; *e*Bioscience, San Diego, CA, USA), and polymer detection system (MACH 4), were used.

After deparaffinization with xilol and hydration, the antigenic recovery was performed through microwave irradiation. The blades were incubated in citrated buffer 10mH (pH 6.0), in microwave oven (average power), for 10 minutes.[17]

Following natural cooling, the citrate solution was ignored and 02 PBS baths of 5 minutes were performed (10mM of sodium chloride on 0.9% and pH 7.5).

For the block of the endogenous peroxidase, the blades were submerged in 5-minute baths in 100ml of methanol with 1.72 ml of hydrogen peroxide, followed by 02 PBS baths, each lasting 5 minutes.

Next, protein blocking was performed by incubating the blades with the protein blocker for 10-15 minutes at room temperature.

For the application of the primary antibody (Ac), the blades were incubated with specific monoclonal antibody Anti-FoxP3, which was diluted in PBS (proportion of 1:100) for16-18 hours, at 4 degrees centigrade, in a humid chamber.

The following day, after 3 PBS baths of 5 minutes, the reaction resumed, as blades were incubated for 30 minutes with the polymer.

After washing the blades three times in PBS solution (5 minutes), they were then incubated in DAB (Diaminobenzidine) for 5 minutes, developing a tenuous, brownish precipitate.

Next, the blades were immerged in distilled running water, and stained with Harris hematoxylin. After drying naturally, they were set with Canada balsam under cover slips.

A blade from the patient was used as negative control in every reaction, using PBS solution in place of the primary antibody. As positive control, two biopsies of lichen planus were used, which were subjected to the process of the patients' samples.

Immunohistochemical staining was visually quantified by counting FoxP3 positive cells in the dermis of the samples. The cell counts were separately averaged for each sample, giving a rough percentage in proportion to the infiltrate.

Data were statistically analyzed using the R Version 2.13.1 (07/08/2011) software package, as follows:

Description of quantitative variables as means ± SD, medians and ranges, and description of qualitative variables as numbers and percentages.

Student's t-test, one-way ANOVA test and non-parametric tests (Kruskal-Wallis and Wilcox).

Pearson correlation study.

Probability or P value of <0.05 was considered statistically significant.

This study was approved by the Clinical Research Ethics Committee of the Alfredo da Matta Foundation.

RESULTS

Patients were classified as follows: (1) I: 9 patients; (2) TT: 13 patients; (3) BT: 26 patients; (4) BB: 3 patients; (5) BL: 8 patients; (6) LL: 27 patients; (7) RR: 8 patients; and (8) ENL: 2 patients. All cases were classified according to the Ridley and Jopling classification.[15,16]

Microscopic examination of the immunohistochemically stained sections revealed FoxP3-positive cells in 96 (100%) of the specimens with an average of 2.82% of the infiltrate (range: 1-7). The immunohistochemical study was performed in all cases; no case was excluded (Table 1).

Table 1: Mean FoxP3 positivity

#	Classification	Overall Mean FoxP3 Positivity	#	Classification	Overall Mean FoxP3 Positivity (%)
1	I	2	49	BB	3
2	I	3	50	BB	2
3	I	1	51	BB	2
4	I	3	52	BL	3
5	I	4	53	BL	1
6	I	2	54	BL	2
7	I	2	55	BL	2
8	I	1	56	BL	5
9	I	2	57	BL	4
10	TT	5	58	BL	5
11	TT	2	59	BL	1
12	TT	2	60	LL	2
13	TT	4	61	LL	1
14	TT	1	62	LL	1
15	TT	2	63	LL	3
16	TT	5	64	LL	5
17	TT	1	65	LL	2
18	TT	3	66	LL	5
19	TT	3	67	LL	2
20	TT	4	68	LL	5
21	TT	5	69	LL	2
22	TT	3	70	LL	1
23	BT	4	71	LL	3
24	BT	2	72	LL	2
25	BT	1	73	LL	4
26	BT	3	74	LL	1
27	BT	2	75	LL	3
28	BT	4	76	LL	2
29	BT	5	77	LL	1
30	BT	2	78	LL	2
31	BT	1	79	LL	2
32	BT	4	80	LL	1
33	BT	4	81	LL	4
34	BT	2	82	LL	4
35	BT	2	83	LL	2
36	BT	1	84	LL	5
37	BT	5	85	LL	4
38	BT	2	86	LL	3
39	BT	2	87	RR	5
40	BT	3	88	RR	2
41	BT	2	89	RR	7
42	BT	4	90	RR	5
43	BT	2	91	RR	3
44	BT	1	92	RR	2
45	BT	5	93	RR	4
46	BT	4	94	RR	5
47	BT	2	95	ENL	2
48	BT	2	96	ENL	3

In TT and BT, FoxP3-positive cells were observed both inside and around the granulomas (Figures 1-2). In BL and LL, FoxP3-positive cells were present randomly in the diffuse macrophages infiltrate (Figures 3-4). A similar pattern was observed in ENL lesions (Figures 5-6). FoxP3-positive cells presented a nuclear staining (Figures 7-8).

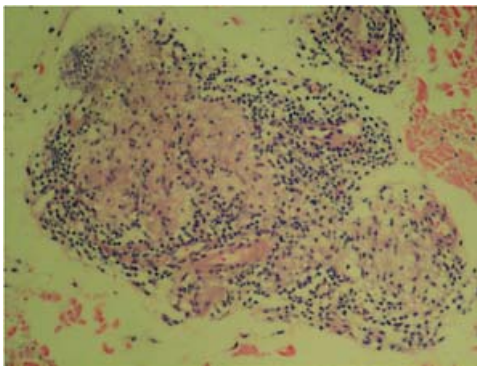

Figure 1: BT leprosy: epithelioid granulomas surrounded and infiltrated by lymphocytes. Hematoxylin–eosin staining; original magnification x100

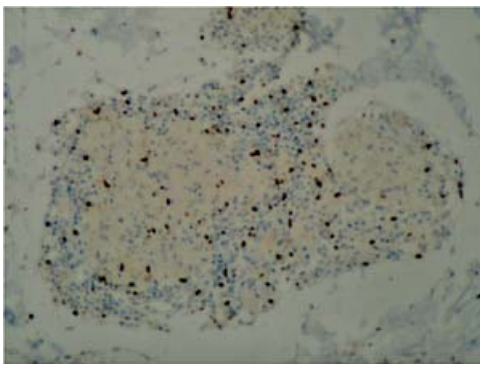

Figure 2: Immunohistochemical stainings of FoxP3; Tregs are present around and within the granuloma; original magnification x100

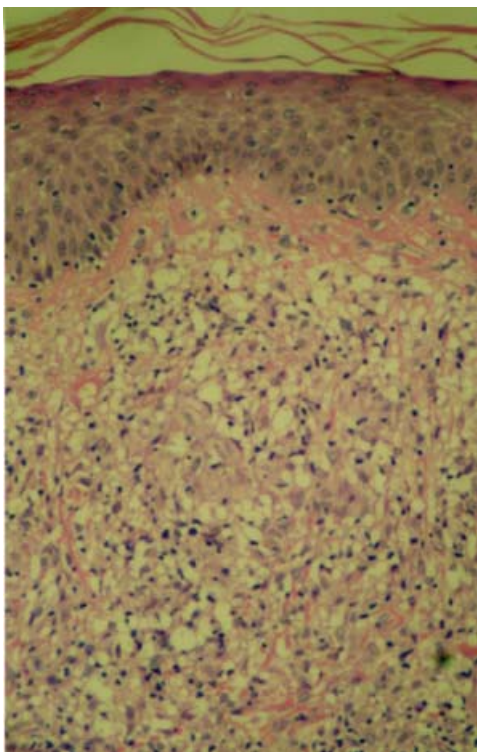

Figure 3: LL leprosy: diffuse infiltrate of vacuolated macrophages together with lymphocytes in the dermis. Hematoxylin-eosin staining; original magnification x100

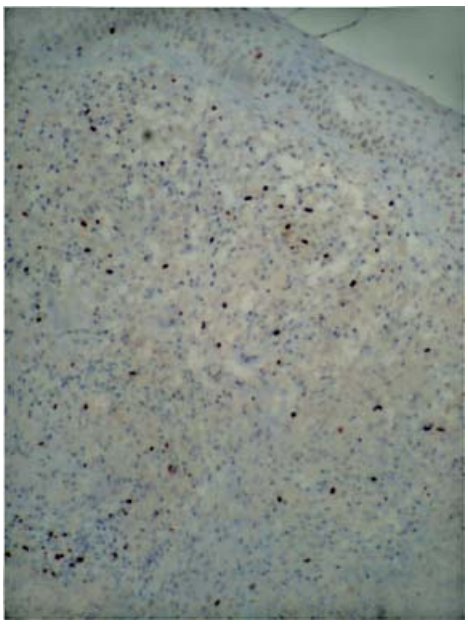

Figure 4: Immunohistochemical stainings of FoxP3 show a diffuse distribution of Tregs; original magnification x100

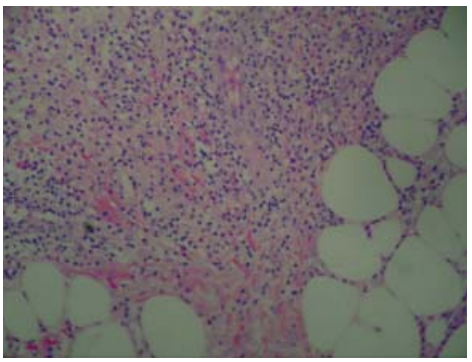

Figure 5: ENL: granulomatous infiltrate of vacuolated macrophages and neutrophils in the dermis. Hematoxylin–eosin staining; original magnification x100

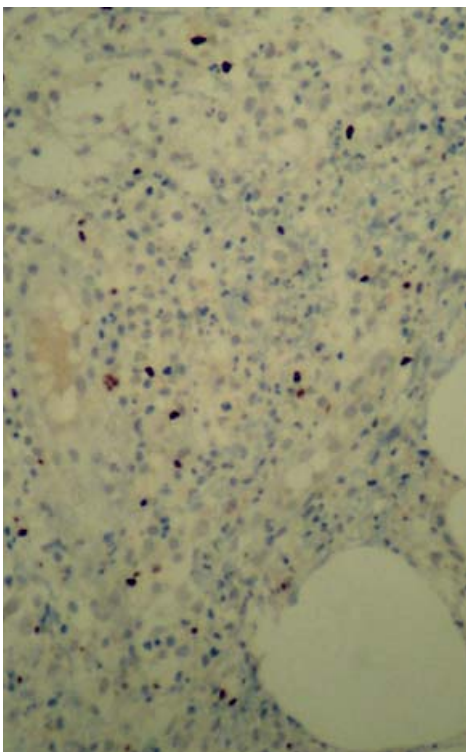

Figure 6: Immunohistochemical stainings of FoxP3 show Tregs distribution in the infiltrate; original magnification x100

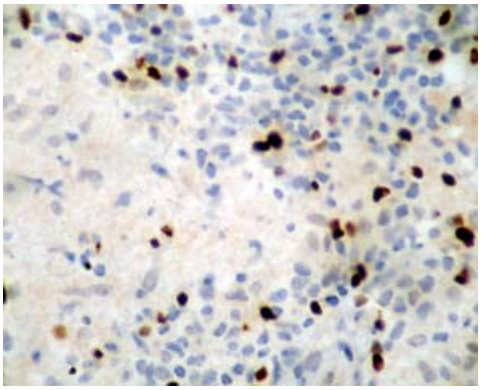

Figure 7: Nuclear FoxP3 staining in BT leprosy. Original magnification x400

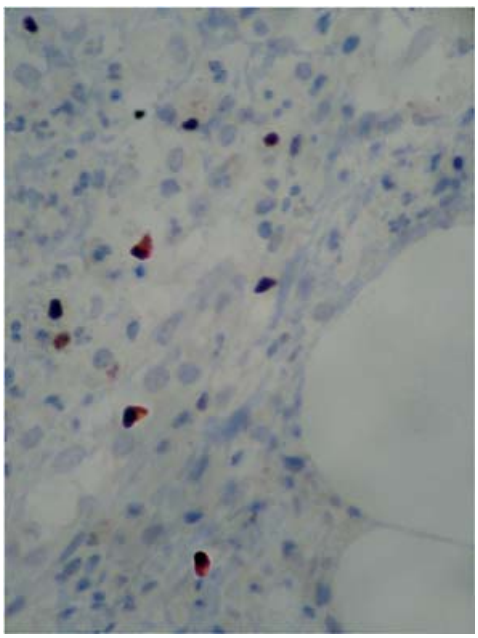

Figure 8: Nuclear FoxP3 staining in ENL. Original magnification x400

A significant statistical increment of FoxP3 expression between leprosy reversal reactions (RR) was found when compared with patients affected by I (P = 0.0228); BT(P = 0.0351) and LL (P = 0.0344), respectively. However, no significant difference was observed between the other clinical forms.

DISCUSSION

Susceptibility or resistance to *M. leprae* infection depends on both innate resistance (mediated by cells of the monocytic lineage) and the degree of specific cellular immunity and delayed hypersensitivity generated by the infected subject. The specific cellular immunity is mediated primarily through the function of T lymphocytes, in cooperation with antigen-presenting cells.[1]

Interactions among host proteins and bacterial antigens preventing invasion and infection by the bacilli have been associated with many genetic factors, and the high complexity of all these molecular events may explain the wide spectrum of clinical forms of leprosy.[18]

The cytokines profile in the leprosy lesions seems to be related to the functions of Toll-like receptors (TLRs)[4], which, particularly in the case of TLR-2, are activated by lipoproteins of *M. leprae*. The capacity to start the protector answer is directly related to the secretion of IL-12/23, as well as the differentiation of macrophages and dendritic cells.[19]

On the foci of infection by *M. leprae*, dendritic cells recognize the bacilli, producing cytokines (IL-12 or IL-10) and chemokines responsible for determining which type of immune response (Th1 or Th2) is generated against *M. leprae*.[20]

While in LL, there is lack of specific CMI to the pathogen with increased production of IL-10 by monocytes, in addition to a predominant Th2 response with release of different subsets of cytokines (IL-4, IL-5, IL-10, and IL-13), the TT pole is characterized by a Th1 immunity with a significant IL-2 production, interferon-gamma (IFN-γ), and TNF-alpha (TNF-α).[12]

Various genes and regions in the human genome have been linked to, or associated with, susceptibility to leprosy *per se* or a particular type of leprosy.[3]

Numerous non-HLA variants located in different genes, such as the vitamin D receptor (VDR), natural resistance-associated macrophage protein 1 (NRAMP1), IL-10 and the PARK2 and PACRG genes, have been described as leprosy genetic risk factors.[4]

Immunopathological studies in HD showed that TT lesions have a predominant CD8+ suppressor-cytotoxic T-cell infiltrate in the mantle of the granulomas, whereas CD4+ T cells have been exclusively observed within the epitheliod granulomas[1]. Lepromatous lesions showed a diffuse distribution of both CD8+ and CD4+ cells among histiocytes, without any semblance of mantle formation.[1]

Other studies noted that, in ENL, the lymphocyte subsets were diffusely distributed throughout the granulomas in a manner similar to LL, whereas with type I reaction, only 30% of the granulomas showed CD8+ T cells located in the mantle zone.[1,21]

Unlike HIV-negative patients with leprosy, patients co-infected with HIV and leprosy present an almost exclusive CD8+ cytotoxic infiltrate at both tuberculoid and lepromatous poles of the disease.[22]

Natural Tregs (CD25+FoxP3+cells) are known to maintain tolerance, suppressing the function of autoreactive T cells in different cutaneous diseases.[22]

They have been found to be absent from, or present at reduced levels, in the skin biopsies of patients with autoimmune diseases like lupus erythematosus and dermatomyositis, of patients with atopic dermatitis and graft versus host disease.[23,24]

The expression FoxP3 was evaluated on a variety of cutaneous infiltrates of T lymphocytes, and it

was found that the Tregs were present in larger amounts under reactive conditions (which barely exceeded 30% of all the infiltrate) than in lymphomas of T-cells, suggesting that the reduction in the regulatory function of the T-cell may be permissive to neoplastic transformation.[1]

Tregs also play an essential role in controlling the excessive immune answer against microbial antigens, especially against pathogens that establish persistent infections.[25]

In this study, FoxP3-positive cells were present in 100% of investigated skin specimens; the density of FoxP3-positive cells was low (average 2.82%, range: 0-7). In TT and BT, FoxP3-positive cells were observed on the center and the periphery of the granulomas (Figures 1 and 2). In BL and LL, FoxP3-positive cells were present randomly in the diffuse macrophages infiltrate (Figures 3 and 4). A similar pattern was also observed in ENL lesions (Figures 5 and 6). In all investigated cases, FoxP3-positive cells were closely related to epitheliod cells or macrophages, suggesting a possible functional interaction between Tregs and histiocytes, as shown in a previous study (Figures 7 and 8).[1]

We observed a statistically significant increment of FoxP3 expression between leprosy reversal reactions (RRs), compared with patients affected by I, BT and LL, showing partial concordance with a recent study, which noted a significant statistical difference in patients with reverse reaction states (BT-RR and BB-RR), compared with the non-reaction forms of the disease (BL, BT, LL, TT).[1]

The data presented here suggest that Tregs may have a relevant role in the etiopathogenesis of leprosy, similar to previous observations concerning leishmaniasis by *Leishmania major*, tuberculosis, Helicobacter pylori infection, Hepatitis B and HIV viroses.[1,26,27]

Tregs appear to control *L. major* infections by modulating the effector immune response via IL-10, TGF-β and immunosuppression[28]. In genetically resistant mouse strains, they down-regulate protective Th1 responses, allowing for parasite survival and maintenance of memory responses.[28]

Tregs increased during active tuberculosis. Depletion of CD4+CD25+ cells improved T cell IFN-γ production by CD4 T cells from newly diagnosed TB patients, indicating that increased frequencies of CD4+CD25+ T cells are not simply a reflection of the excessive immune activation during TB, but that subsets, presumably those with a CD4+CD25hi+ phenotype, indeed have immunoregulatory properties.[29]

This study may have a certain limitation due in that we sought FoxP3 positive cells in skin biopsies. The ideal design would have been to analyze peripheral blood and skin samples from the same patient.[30]

A control-case study using flow cytometry, involving 38 leprosy cases (9 TT, 6 BT, 4 BL, 8 LL, 3 BB and 6 erythema nodosum leprosum) and 38 controls, indicated an increase in Tregs and FoxP3 expression in all forms when compared with controls, mainly TT. This study suggests that the increase in Tregs could benefit leprosy patients, for the immune answer would tend to the cellular pole of the disease.[12]

This study has also demonstrated a reduction in Tregs and an increase in FoxP3 expression, in the cases of erythema nodosum leprosum and LL, suggesting that the high FoxP3 expression induces a heavier cellular immune suppression, leading to the humoral, lepromatous pole of the disease.[12]

Recently, another control-case study using flow cytometry and immunohistochemistry, involving 28 leprosy cases (2 TT, 10 BT, 13 BL, 3 LL) and 6 controls, showed 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 individuals capable of limiting *M. leprae* growth (tuberculoid leprosy).[13] Increased frequency of CD25+ FoxP3+ T cells was seen both in *in vitro M. leprae*-stimulated and disease sites.[13] The results differ from those of another group, which found higher frequency of circulating Tregs in the peripheral blood of tuberculoid patients compared with lepromatous patients.[12]

No consensus exists in the literature on the role of Tregs in leprosy. Common knowledge suggests that Tregs may alter Th1 and Th2 response, interfering with the immune response against mycobacterial infection.[6]

Both the production and the fact that Tregs depend on an available cytokines milieu, means that the microenvironment plays a crucial role in their differentiation, expansion and function. The development and maintenance of CD4+CD25 high FoxP3+ cells depends on TGF-β.[12,31] IL-2 is essential for maintaining balanced, natural and adaptive Treg activity.[32] In addition, IL-2 promotes expansion and FoxP3 expression in Tregs, both *in vivo* and *in vitro*.[12,33] Further, previous studies have demonstrated that the strength of T cell receptor and IL-2 signals influence the magnitude of suppression achieved by Tregs, and that IL-2 plays an important double-edged role both in enabling Tregs to suppress and target cells to escape Tregs-suppression.[34]

This suggests that the expansion or detection of Tregs in the leprosy tuberculoid or lepromatous poles, as shown previously, is probably due to the specific cytokines profile of these patients.[12]

Our results confirm the previous findings of another study[1], demonstrating that there is a statistically significant difference in FoxP3 expression in cases of leprosy reversal reaction, compared with patients of non-reaction leprosy (I, BT, LL). These data

suggest that Tregs may have a relevant role in the etiopathogenesis of leprosy, particularly in type 1 reaction, similar to previous observations concerning leishmaniasis by *L. major* and tuberculosis by *M. tuberculosis*.[25,26]

CONCLUSION

1) Tregs in leprosy and its reactional states have been investigated in few studies and they show divergent results.

2) These studies utilized a small number of cases with restricted clinical forms.

3) This study involved 96 cases of all the clinical forms of leprosy and showed that Tregs have a relevant role in the etiopathogenesis of leprosy, particularly in type 1 reaction; similar to previous observations concerning leishmaniasis by *L. major* and tuberculosis by *M. tuberculosis*.

4) Further studies are needed for a more complete understanding of the role of Tregs in leprosy, which could lead to new therapeutic approaches. ❑

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