Tropical medicine rounds

Circulating CD4$^+$ CD25^{high}FoxP3$^+$ T cells vary in different clinical forms of leprosy

Enas A. S. Attia[1], MD, Marwa Abdallah[1], MD, Abeer A. Saad[2], MD, Ashraf Afifi[1], MD, Alaa El Tabbakh[1], MD, Dina El-Shennawy[2], MD, and Hala B. Ali[2], MD

[1]Department of Dermatology, Venereology and Andrology, [2]Department of Clinical Pathology, Faculty of Medicine, Ain Shams University, Cairo, Egypt

Correspondence
Enas Attia, MD
Dermatology, Venereology and Andrology, Faculty of Medicine, Ain Shams University, Cairo, Egypt
E-mail: annosah74@hotmail.com

Abstract

Background CD4$^+$ CD25^{high}FoxP3$^+$ regulatory T cells (T-regs) were reported to increase in chronic infections. We aimed at studying their frequency in leprosy to investigate their role during *Mycobacterium leprae* infection.

Methods Using flow cytometry, the frequency and FoxP3 expression of circulating T-regs was assessed in 38 leprosy patients and 38 healthy controls. Patients were divided into; group I tuberculoid (TT), group II borderline cases [borderline tuberculoid (BT), borderline (BB), and borderline lepromatous (BL)], group III lepromatous (LL), and group IV erythema nodosum leprosum (ENL).

Results Mean T-regs% and FoxP3 expression were significantly elevated in patients (particularly TT) compared to controls (3.8 ± 2.5% vs. 2.5 ± 0.8% and 78.8 ± 56.2% vs. 55.8 ± 15.7%, respectively) ($P < 0.05$). Comparing the four disease groups, T-regs% was significantly different (median 5.3% in group I, 3.4% in group II, 2.8% in group III, and 1.2% in group IV; $P = 0.005$). FoxP3% on T-regs was not significantly different between them [median 71.5% in TT, 62.3% in borderline categories, 67.75% in LL, and 85.75% in ENL; $P = 0.149$). Notably FoxP3 expression was significantly higher in ENL than controls ($P = 0.011$).

Conclusion The frequency and suppressive marker of circulating T-regs are elevated in TT patients. Patients with LL and ENL express significantly lower frequency of T-regs and higher FoxP3 expression (in ENL), consistent with disease progression and immune hyperactivation in these disease categories. Thus, rather than being detrimental to immunity, intact T-regs activity may be beneficial to leprosy patients.

Introduction

Leprosy is a chronic granulomatous disease, caused by *Mycobacterium leprae*, principally affecting peripheral nerves and skin. The pathogenesis and thus the clinical features reflect variable degree to which cell-mediated immunity (CMI) is expressed.[1] Lepromatous leprosy (LL) represents a failure of CMI specifically towards *M. leprae*, with absence of activated lymphocytes and macrophage, meaning that nerve damage is slow and gradual. In tuberculoid leprosy (TT), CMI is strongly expressed so that the infection is restricted to one or a few skin sites and/or peripheral nerves. Between those two polar forms lie the borderline forms of the disease, with the extent of the disease reflecting the balance between CMI and the bacillary load.[2] Borderline patients; borderline tuberculoid (BT), borderline (BB), and borderline lepromatous (BL), are immuologically unstable and at risk of developing type 1 (reversal) reactions which are delayed hypersensitivity reactions caused by increased recognition of *M. leprae* antigens in skin and nerves.[3] On the other hand, type 2 reactions; erythema nodosum leprosum (ENL) are due to immune complex deposition and occur in BL and LL patients.[2]

Forkhead box P3 (FoxP3) is a transcription factor associated with functional regulatory T cells, known as T-regs. Although recent studies have revealed many different types of T-regs, both naturally occurring and inducible, FoxP3 has been shown to have a direct role in inducing immunosuppression and has been identified as a good marker for cells with a suppressor function.[4–7] In humans, these cells were first thought to be specifically CD4$^+$ CD25^{high} naturally occurring T-regs, but more recent studies have shown this not to be the case and FoxP3 is also expressed in other cells (such as CD8$^+$) with a suppressor function.[8–10] Although T-regs that express FoxP3 are involved in the beneficial attenuation of

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immunopathology,[11] they are also implicated in down-regulation of protective responses to infection.[12,13]

The immune response to infection represents a complex balance between the successful induction of pro-inflammatory anti-pathogen responses and anti-inflammatory responses required to limit damage to host tissues. T-regs undoubtedly play an important role in controlling this balance during infection, and the results can range from being highly detrimental to the host to highly beneficial to both host and pathogen.[14]

Recently, one mycobacterial infection with depressed T cell function, tuberculosis, was found to involve expansion of $CD4^+$ $CD25^{high}$ T-regs.[15–17] Therefore, we aimed to study the frequency and suppressive marker of circulating T-regs in leprosy and compare their level in different types of leprosy, in order to gain further insight into their role in the immune response during *M. leprae* infection.

Subjects and methods

Subjects

Untreated leprosy patients were enrolled from those attending El Qual'aa Hospital for Dermatology and Leprosy, in Cairo, Egypt over a period of 10 months. Patients were evaluated according to clinical examination, skin smear examination, and histopathological examination, and were divided into 4 groups: group I patients with TT, representing the immunocompetent group; group II patients with BT, BB and BL representing moderate CMI; group III patients with LL representing low CMI patients; and group IV patients with ENL representing patients with low CMI in reaction.

Patients who started antileprotic treatment or were on any kind of immunomodulatory or immunosuppressive therapy likely to alter the results of the study, such as systemic corticosteroids, were excluded.

Age-matched subjects with negative hepatitis C virus (HCV) and hepatitis B virus (HBV) serology and negative tuberculin test from Ain Shams University Hospital staff members and personnel, were also included in the study, comprising the control group.

Methods

Two milliliters of peripheral venous blood were collected from each subject using vacutainer containing anticoagulant potassium ethylene diamine tetraacetate (EDTA) in a final concentration of 1.5 mg/ml.

Fifty microliters of whole anticoagulated blood was lysed using 1 ml IQ test lysing reagent (Beckman Coulter, Miami, USA) followed by washing with phosphate buffer saline (PBS) (Oxoid, Hampshire, UK). After that, the cells were stained with combinations of the following antibodies (5 μl each): anti-CD25-PE, anti-CD4-FITC, anti-CD8–PE-Cy5 and isotype controls (FITC, PE and PE-Cy5) (Beckman Coulter). The test tubes were then incubated in dark for 20 min followed by washing with PBS.

Intracellular staining FoxP3-PE-Cy5 (eBioscience, California, USA) was as follows: anticoagulated whole blood was fixed and permeabilized using FoxP3 Staining buffer Set (eBioscience) according to the manufacturer's instructions with certain modifications, in brief:

After washing, the cell pellet was resuspended in 0.5 ml of freshly prepared fixation/permealization working solution and incubated for 30 min at 4°C in dark. This was followed by washing once with PBS followed by washing once again with 1 ml of 1 × permealization buffer. Ten microliters of FoxP3 or isotype control were added and incubated for 30 min at 4°C in dark. Lastly washing with PBS followed by resuspension in PBS for analysis was done.

Data acquisition and analysis were performed on EP-ICS XL flow cytometry using SYSTEM II version 3 software with a standard three-color filter configuration.

Lymphocytes were gated via their forward and side scatter properties, and T cells were identified based on their expression of CD4.

To discriminate between $CD25^{high}$ T-regs and $CD25^{low}$ activated effector-memory T cells (T-effs), we used CD25 expression on CD8+ cells as an internal control. CD8+ cells only express intermediate levels of CD25 ($CD25^{low}$), whereas $CD4^+$ T cells express CD25 with high ($CD25^{high}$) or intermediate ($CD25^{low}$) intensities. Only $CD4^+$ cells expressing CD25 with higher intensities than the CD8+ cells were included in the gate for $CD25^{high}$ cells. The gate for $CD25^{low}$ cells was set to include cells expressing CD25 at levels above those of the isotype control but at lower expression levels than the $CD25^{high}$ cells, according to Lundgren et al.[18] (Fig. 1a–d).

Statistical analysis

Data were analyzed statistically by IBM computer using SPSS (Statistical Program for Social Science version 12) (SPSS Inc., Chicago, IL, USA) as follows:

- Description of quantitative variables as means ± SD, medians and ranges, and description of qualitative variables as numbers and percents.
- Student's *t*-test, one-way ANOVA test and non parametric tests (Mann–Whitney and Kruskal–Wallis).
- Pearson correlation study.

Probability or P value of <0.05 was considered statistically significant, while P value ≤0.001 was considered statistically highly significant.

Results

Thirty-eight patients with different clinical forms of leprosy [23 males and 15 females; age range 13–60 years (mean 36.55 ± 13.48 years)], and 38 matched controls [17 males and 21 females; age range 23–63 years (mean

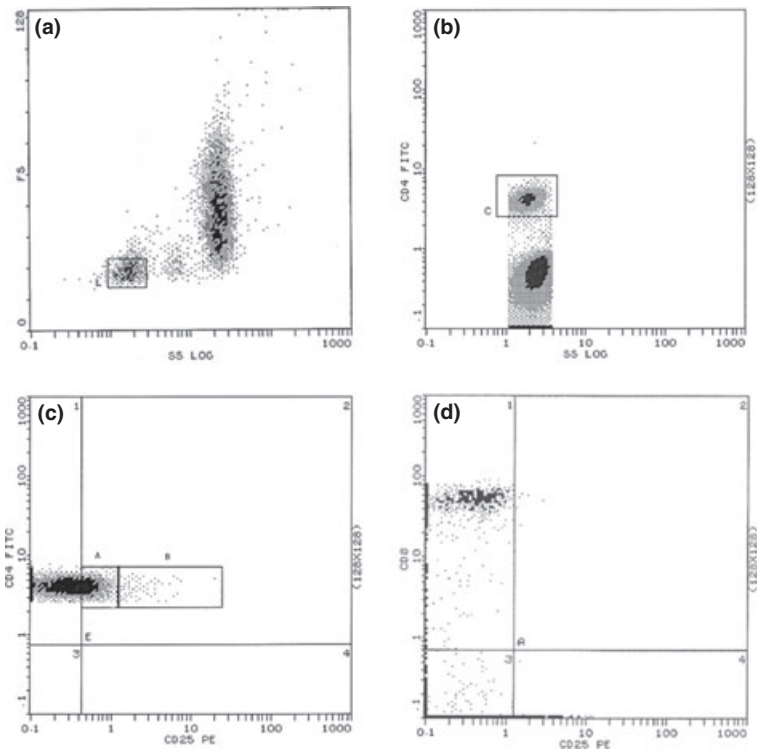

Figure 1 The gating strategy; CD4+ cells were acquired after gating the lymphocyte population by forward- and side-scattered properties. Gating approach for discrimination of CD25^{high} (T-regs) and CD25^{low} (T-effs) cells were set using CD25 expression levels on CD8+ cells (low expression only), as described in "Subjects and methods" section

43.76 ± 10.17 years)] were included in the present study. Overall disease duration ranged from 10 days to 17 years. Group I comprised 9 TT patients; group II, 13 patients (BT = 6, BB = 3, BL = 4); group III, 8 LL patients; and group IV, 6 ENL patients. The remaining two patients presented with type 1 leprosy reaction and were not included in the statistical analysis of clinical forms.

To ensure proper gating process, we compared mean FoxP3 expression% in all the study population (patients and controls), in T-regs versus T-effs, which showed a statistically highly significant difference, i.e., proper gating (Fig. 2).

Patients vs. controls

As demonstrated in Table 1, comparing the absolute T-reg count and T-reg% in patients versus controls revealed statistically significant higher values among patients (P = 0.049 and 0.024 respectively).

Regarding CD4$^+$ CD25^{low} T effector cells% (T-effs%) and absolute number, statistically significant elevation in patients than controls was found ($P < 0.001$ and P = 0.009 respectively).

The suppressive marker; FoxP3 expression% on T-regs was also statistically higher in patients than in controls (P = 0.002).

Although the median of T-regs/T-effs ratio was lower in patients compared to controls, the difference was not statistically significant (P = 0.791) (Table 1).

Patients' results

In all patients, total leukocytic count was within normal range except in group IV patients and the two patients who presented with type 1 reaction, i.e., all reactional leprosy patients. In these patients total leukocytic count was elevated with a range of 12.9 to 26.5 × 10^3/μl and a mean of 16.6 × 10^3/μl, and all had absolute neutrophilia.

T-regs%, absolute count, and FoxP3 expression% did not correlate with disease duration [(r = 0.071, P = 0.67), (r = 0.032, P = 0.851), and (r = 0.07, P = 0.678) respectively] (data not shown).

Comparison between different clinical forms of leprosy (Table 2) revealed statistically significant differences in T-reg% (P = 0.005), and absolute T-reg count (P = 0.01). Group III (LL) showed significantly lower T-reg% than group I (TT) (Z = 2.651; P = 0.006). Group IV (ENL) showed significantly lower T-reg% than all other leprosy groups; I, II, and III (Z = 2.715; P = 0.005, Z = 2.591; P = 0.007, and Z = 1.941; P = 0.052 respectively).

Regarding the absolute T-reg count, group IV showed significantly lower count than all other leprosy groups; I, II, and III (Z = 2.711; P = 0.005, Z = 2.458; P = 0.012, and Z = 2.711; P = 0.005 respectively). However, FoxP3 expression% on T-regs was not statistically different among different groups.

Although T-effs% and absolute number were not statistically different among various clinical forms of leprosy,

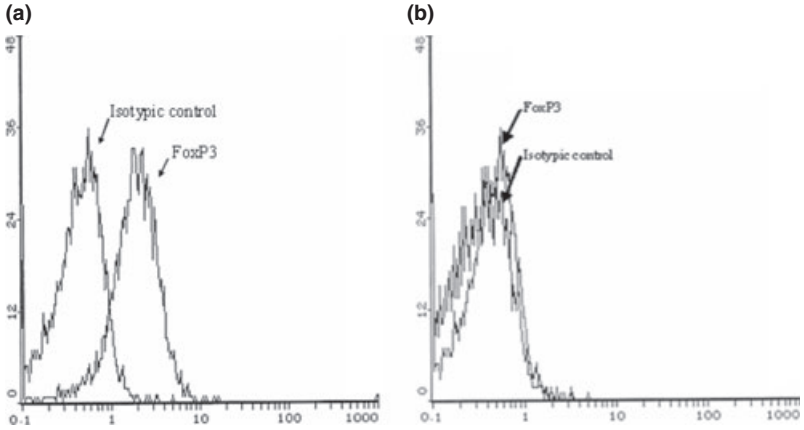

Figure 2 (a) FoxP3 expression on T-reg cells. (b) FoxP3 expression on T-eff cells

Table 1 Comparison between patients and controls

	Patients	Controls	*Z or T*	*P* value
T-regs%	3.8 ± 2.5	2.5 ± 0.8	*Z* = 2.264	0.024*
T-regs absolute no./μl	78.8 ± 56.2	55.8 ± 15.7	*Z* = 1.971	0.049*
T-regs FoxP3 %	68 ± 12.8	60 ± 7	*T* = 3.356	0.002*
T-effs%	12.9 ± 6	9.3 ± 2.1	*T* = 3.406	<0.001**
T-effs absolute no./μl	285 ± 139.8	227.6 ± 98.3	*T* = 2.07	0.009*
T-effs FoxP3 %	18.7 ± 9.7	13.7 ± 2.3	*T* = 3.075	0.003*
T-regs/T-effs (median)	0.24	0.36	*Z* = 0.265	0.791

*P < 0.05 significant difference.
**P < 0.001 highly significant difference.

the median T-regs/T-effs ratio showed a statistically significant difference between the four groups (P = 0.034) (Table 2). Group IV (ENL) showed significantly lower ratio than group I (Z = 2.475; P = 0.012).

The two patients with BL who presented with type 1 (reversal) reaction had the following findings: one patient had T-regs% of 4.5, their absolute number was 92 cells/μl, and FoxP3 expression% was 80.9, while T-effs% was 21, their absolute number was 433 cells/μl, and FoxP3 expression% was 15.2. In the other patient, T-regs% was 12.6, their absolute number was 267 cells/μl, and FoxP3 expression% was 55.3, while T-effs% was 9.7, their absolute number was 205 cells/μl, and FoxP3 expression% was 18.8.

Disease forms versus controls

Comparison between different clinical forms of leprosy to controls revealed statistically significant differences as regards the studied parameters; T-reg%, T-reg count, T-reg FoxP3% expression, T-effs%, and T-regs/T-effs ratio (P = <0.001, 0.002, 0.011, 0.035, and 0.048 respectively) (Table 2).

Group I (TT) showed significantly higher T-reg%, T-reg count, T-reg FoxP3 expression, and T-effs% than controls (P = <0.001, 0.007, 0.005, and 0.015 respectively) (Table 3).

On the other hand, group IV (ENL) showed significantly lower T-reg%, and T-reg count, with significantly higher T-reg FoxP3% expression and T-effs% than controls (P = 0.022, 0.009, 0.033, and 0.003 respectively) (Table 3). They also showed statistically significant lower T-regs/T-effs ratio than controls (P = 0.01) (Table 3), as a reflection of depressed patients' T-reg count together with elevated T-effs number.

Table 2 Comparison between median values of different clinical forms of leprosy, and between them and control values, using Kruskal–Wallis test

	Group I	Group II	Group III	Group IV	χ^2	*P* value	Control	χ^2	*P* value
T-regs%	5.3	3.4	2.8	1.2	12.852	0.005*	2.5	20.214	<0.001**
T-regs absolute no./μl	82.0	67.0	62.0	35.5	11.305	0.010*	50.0	16.972	0.002*
TregsFoxP3% expression	71.5	62.3	67.75	85.75	5.336	0.149	56.2	13.144	0.011*
T-effs%	11.3	12.4	8.9	14.7	2.629	0.452	9.0	10.354	0.035*
T-effs absolute no./μl	232.0	306.0	308.0	320.0	0.977	0.807	203	5.913	0.206
T-regs/T-effs	0.38	0.27	0.22	0.08	8.696	0.034*	0.36	9.604	0.048*

*P < 0.05 significant difference.
**P < 0.001 highly significant difference.

Table 3 Comparison between median values of each clinical form of leprosy, and median values of controls, using Mann–Whitney test

	Control	Group I	Z	*P* value	Group II	Z	*P* value	Group III	Z	*P* value	Group IV	Z	*P* value
T-regs%	2.5	5.3	3.533	<0.001**	3.4	2.074	0.022*	2.8	0.568	0.579	1.2	2.257	0.022*
T-regs count/μl	50.0	82.0	2.631	0.007*	67.0	1.528	0.126	62.0	1.686	0.096	35.5	2.541	0.009*
TregsFoxP3%	56.2	71.5	2.766	0.005*	62.3	1.3	0.193	67.75	1.919	0.057	85.75	2.128	0.033*
T-effs%	9.0	11.3	2.417	0.015*	12.4	1.052	0.293	8.9	0.32	0.765	14.7	2.819	0.003*
T-effs count/μl	203	232.0	1.085	0.291	306.0	0.488	0.626	308.0	1.903	0.059	320.0	1.888	0.061
T-regs/T-effs	0.36	0.38	1.672	0.097	0.27	0.304	0.761	0.22	0.131	0.898	0.08	2.441	0.013*

*$P < 0.05$ significant difference.
**$P < 0.001$ highly significant difference.

Discussion

Several $CD4^+$ T-cell populations have been shown to regulate activation, differentiation, and effector functions of T cells.[19–23] One population is the $CD4^+$ $CD25^+$ cell population which represents 5–10% of human circulating $CD4^+$ T cells, but only the subset expressing high levels of CD25; the interleukin (IL)-2 receptor α-chain, exhibit strong regulatory function.[24] These $CD4^+$ $CD25^{high}$ T cells (T-regs) constitute 1–2% of the $CD4^+$ T-cell population. However, because CD25 is also a marker of activation of T effector cells, FoxP3 has been identified as a specific marker of $CD4^+$ $CD25^{high}$ T-regs, distinguishing them from recently activated, non-regulatory $CD4^+$ $CD25^{low}$ T-eff cells.[25–27]

Leprosy is characterized by a wide spectrum of clinical forms, depending on CMI. While in LL there is lack of specific CMI to the causal organism with increased monocyte production of IL-10, and predominant Th2 response with release of different subsets of cytokines (IL-4, IL-5, IL-10, and IL-13), the tuberculoid pole is characterized by Th1 profile with IL-2, interferon (IFN)-γ, and TNF production.[28]

In this study, data on the frequency of circulating $CD4^+$ $CD25^{high}$ T-regs and their FoxP3 expression in leprosy patients are presented for the first time. We found a higher absolute number and higher frequencies of T-regs, expressing more FoxP3 in leprosy patients compared to controls. We also found a higher absolute number and higher frequencies of $CD4^+$ $CD25^{low}$ effector cells, again expressing more FoxP3 in leprosy patients compared to controls. However, comparing different clinical types of leprosy to controls indicated that these parameters are significantly increased in the TT form. These findings denote active accumulation of both T-regs and T-effs in the peripheral blood of TT leprosy patients.

Studies in infection models described significant immunosuppressive activity of T-regs for antigen-specific T-cell responses against pathogens most relevant in the context of circumstances of antigen persistence described in tuberculosis,[15–17] *H. pylori* infection,[19] leishmaniasis,[21] *HCV* infection,[29] human immunodeficiency virus (HIV) infection,[30] and *HBV* infection.[31] However, a low persistent level of infection, seen with these pathogens, is advantageous in order to generate a memory response. Because memory T cells have a low activation threshold and readily respond to minimal antigenic stimulation,[32] T-regs may protect from non-specific memory T-cell activation and potential tissue damage. T-regs may also be responsible for the fact that memory T-cell population has a slow proliferative turnover and reduced susceptibility to apoptosis, despite being highly susceptible to cytokines and low-affinity antigens.[33,34] Thus, T-regs may actively contribute to the maintenance of memory cell populations, and effective anti-pathogen immune response in leprosy, as evidenced by their significant increase among TT patients.

We demonstrated elevated total leukocytic count and absolute neutrophilia in all reactional leprosy patients. This suggests a preliminary investigative role of total and differential leukocytic count in reactional leprosy patients, provided there is no other concomitant infection or any other possible cause.

Comparing different clinical forms of leprosy, we demonstrated that the frequency of T-regs was decreased in patients with LL, and was even more reduced in those with ENL. Because relatively intact CMI mechanisms are demonstrated towards the tuberculoid pole, expansion of T-regs in tuberculoid patients could be interpreted as a protective counter-mechanism trying to regulate effective anti-pathogen immune response and to attenuate the *M. leprae*-induced chronic immune activation. On the other hand, T-regs depletion towards the lepromatous pole is consistent with disease progression and humoral immune hyperactivation manifested as ENL; a prominent feature in this category of patients.[28] This was supported by our finding of significantly increased suppressive

marker; FoxP3% expression in this category of patients, compared to controls.

Both the generation and fate of T-regs depend on available cytokines, i.e. the microenvironment plays a crucial role in their differentiation, and also in their expansion and function. The development of and maintenance of $CD4^+$ $CD25^{high}$ $FoxP3^+$ cells depends on TGF-β.[35] In addition, IL-2 is essential for maintaining a balanced natural and adaptive T-reg activity.[36] IL-2 also promotes expansion as well as FoxP3 expression in T-regs, both *in vivo* and *in vitro*.[37] Besides, previous studies showed that the strength of T cell receptor and IL-2 signals affects the magnitude of suppression achieved by T-regs, and that IL-2 plays an important double-edged role both in enabling T-regs to suppress and target cells to escape T-regs-suppression.[30]

From the above mentioned data, it is likely that expansion or depletion of T-regs in the tuberculoid pole- or the lepromatous pole-patients, respectively, is due to the specific cytokine profile of these patients. However, detrimental (anti-effector) and protective (anti-immunopathological) roles of T-regs in leprosy remain equally likely, and whether suppressive or regulatory, the detected T-regs in various forms of leprosy need further functional studies.

Our results were extremely variable with reversal (type 1) reactions, as one patient showed extremely high T-regs frequency compared to the other, but with lower FoxP3 expression %. Further investigations, including functional and cytokine profile studies, on larger group of patients with type 1 reaction are needed.

Although T-effs% and absolute number were not statistically different among various clinical forms of leprosy, the median T-regs/T-effs ratio showed a statistically significant difference, being low among LL patients particularly those with ENL, compared to other disease forms, and to controls, as a reflection of depressed patients' T-reg count together with elevated T-effs number. This suggests a negative correlation between T-regs/T-effs ratio and bacillary load, indicating a possible regulatory rather than suppressive role of T-regs in leprosy. While some studies showed a positive correlation between T-regs frequency and infection load,[29,31] others indicated that rather than the number of T-regs, it is the balance or the ratio between T-regs and T-effs that determines the infection load.[29] Elucidation of such role necessitates functional studies on T-regs and T-effs cells, both in the circulation and in lesional skin.

In summary, data presented here indicate that active leprosy (particularly TT) is associated with expansion of $CD4^+$ $CD25^+$ T cells overall, and specifically T-regs subsets, suggesting a possible role in immune responses during *M. leprae* infection. Patients with LL and ENL express significantly lower frequency of T-regs than other clinical forms, but with more suppressive marker expression than normal controls (particularly in ENL), which is consistent with disease progression and immune hyperactivation in these categories. Thus, rather than being detrimental to immunity, intact T-regs activity may be beneficial to leprosy patients. These findings allow a different and novel view on the pathogenesis of leprosy, and may lead to the development of new; hitherto unconsidered, therapeutic strategies in this disease, i.e., drugs that target T-regs. Further studies should be directed at identifying the mediators and mechanisms involved in the immunoregulatory and/or immunosuppressive properties of T-regs during the course of human *M. leprae* infection.

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