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Thymus Output of CD4+CD25highTreg+ is Increased in Patients Carrying Leprae Disease

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Abstract

Background: Leprosy is an infectious disease caused by the intracellular pathogen *Mycobacterium leprae*, which is a microorganism that evades the host's immune response. The mechanisms regulating immune circumvention are not fully understood. It has been proposed that the pathogen alters the mechanisms regulating the immune response of the host to escape immune surveillance. Thus, regulatory T cells are important immune elements that must be investigated. To escape the immune response using regulatory properties or natural regulatory T cells (nTreg), *M. leprae* increases the number of nTregs observed in the steady-state. Because nTregs are thymus derived, one way to verify if *M. leprae* infection induces de novo nTreg cells is to determine the nature of recent thymus emigrant nTregs in leprosy patients and compare the cells to healthy controls.

Objectives: In this study, we (i) quantified the regulatory T cells in the peripheral blood of leprosy patients compared to healthy age-matched controls, (ii) determined whether these cells show a phenotype of recent thymic emigrants, and (iii) evaluated the cytokine profile in plasma samples. All of the studies were performed using flow cytometry.

Results: We found higher levels of nTreg cells during early periods of the disease. Additionally, there was a continuous decrease in nTreg cells throughout the treatment. Moreover, the nTregs were recent thymic emigrants, as shown by the expression of a specific marker (PTK7).We did not find any difference in the cytokine profile when comparing patients and controls. Conclusion: The levels of CD4+CD25highFoxp3+ T cells in patients with leprosy are most likely evidence of thymic output. In addition, blood levels of CD4+CD25highFoxp3+PtK7+ can be used to monitor treatment progress in leprosy.

Keywords: Tregs; Leprosy; Ptk7

Introduction

Leprosy is a chronic disease caused by *Mycobacterium leprae*. This disease is characterized by skin lesions and neural damage that frequently result in deformities and incapacitation [1]. The pathogenesis and clinical features of the disease are due to the variable response of cell-mediated immunity. Both Mycobacterium load and clinic pathological manifestations influence the host immune response. Thus, while there is a strong cell-mediated immunity toward paucibacillary (tuberculoid) polar and borderline forms of leprosy, there is low cell-mediated immunity toward multibacillary lepromatous polar and borderline forms [2].

Regulatory T cells (Tregs) are key players in dampening the potential harmful effects of immune responses by maintaining immunological homeostasis and preventing autoimmune diseases [3, 4].

These T cells are also involved in the down regulation of immune responses to infectious agents. Given the extraordinary diversity of mechanisms that infectious agents have evolved to evade destruction by the immune system [5], it is not unexpected that various microorganisms have targeted the suppressive nature of regulatory T cells as a mechanism of immune evasion. The basic immunological principle is that constant environments tend to be tolerated, whereas changing environments elicit reactions [6].

An infectious agent can stimulate an immune response but can evade complete eradication and establish itself in a niche where it does not elicit inflammatory responses. When this occurs, the immune system may adapt to the infectious agent as if it were a self-antigen. Recent findings suggest that the induction of partial tolerance through regulatory T cells is a common mechanism used by chronic microorganisms to evade immunological destruction. This has been observed for *Borrelia burgdorferi* [7], *Schistosomiasis mansoni* [8], and *Schistosomiasis haematobia* [9], as well as in human immunodeficiency viral infections [10].

Because there are natural regulatory T cells (nTregs) [11] and induced regulatory T cells (iTregs) [12], it is important to address whether regulatory T cells that develop during infectious disease are generated de novo from the thymus (nTregs). Alternatively, the Tregs could also be peripherally expanded in response to antigen from the pathogens (iTregs).

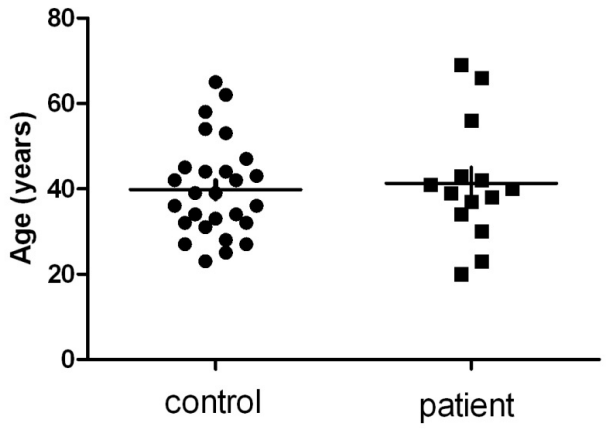

Figure1: Patients and healthy controls were compared by age, and no differences between the two groups were found.

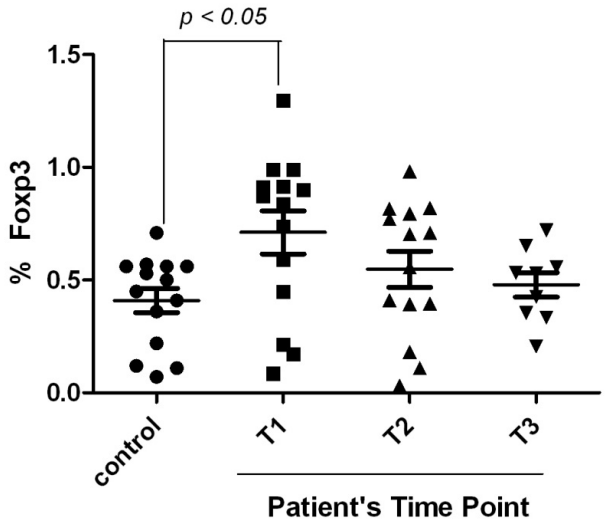

Figure 2: Age-matched patients and controls were compared for CD4+CD25highFoxp3+T cell numbers in peripheral blood. Patients were sampled at three different times (T1, T2 and T3). The controls were sampled once. A significant difference was found between controls and patients at the first time point (T1). There were no differences at the later time points (T2 and T3). A continuous decrease in CD4+CD25highFoxp3+T cells number was found throughout treatment.

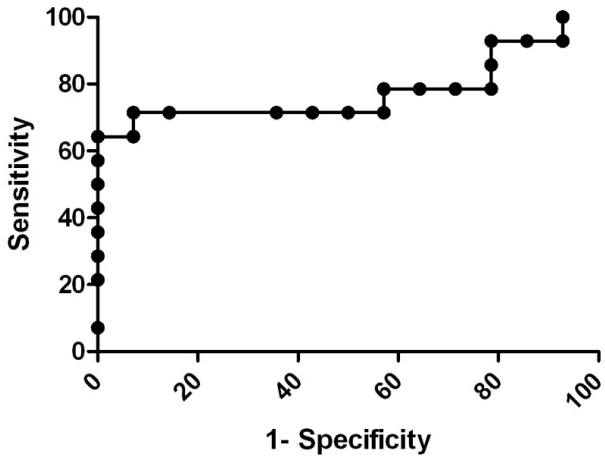

Figure 3: CD4+CD25highFoxp3+T cells number is not a suitable parameter to discriminate between leprosy patients and controls based on the ROC curve.

One way to answer this question is to perform a screen of regulatory T cells (CD4+CD25highFoxp3+) for a suitable marker for Recent Thymic Emigrant (RTE). Protein tyrosine kinase 7 (PTK7) is a transmembrane receptor that regulates morphogenetic processes, and it has been recently suggested as an RTE marker [13]. Here, we evaluated whether the previously reported increase in the frequency of regulatory T cells in patients with leprosy [14-16] is due to peripheral expansion of iTreg or augmented thymus output of nTregs.

Materials and Methods

Samples and participants

Patients and healthy controls were enrolled in this study from July 2012 to April 2013 at the Getúlio Vargas Hospital in Teresina, Brazil. The healthy control group was composed of 27 subjects from Piauí state (age ranging from 18 to 74 years, median=42 years) containing 15 females and 12 males. Each component of the group was a regular blood donor previously vaccinated with BCG and negative for leprosy. The leprosy patients (age ranging from 18 to 80 years, median=40 years) had either paucibacillary (PB=03 subjects) or multibacillary (MB=07 subjects) clinical forms of the disease. The paucibacillary forms were characterized by the presence of up to five characteristic lesions, no affected nerves and negative bacilloscopy.

The multibacillary patients presented more than five characteristic lesions or showed positive bacilloscopy and/or at least one compromised nerve trunk [17]. The study inclusion criteria for patients were the following: laboratory and clinical diagnosis of leprosy including both type 1 or 2 reactions, both male and female patients, any race or color, age range from 18 to 80 years, lucid and freely agreeing to participate in the study.

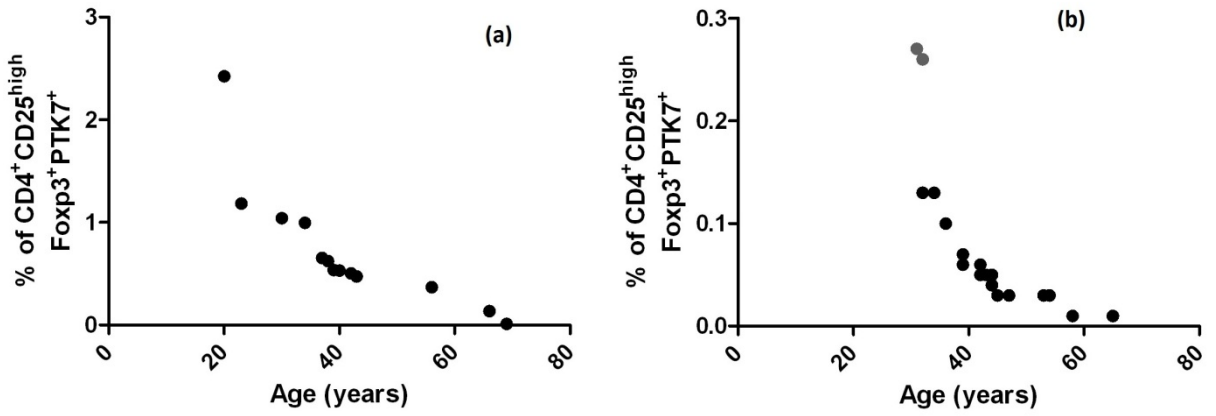

Figure 4: PTK7 levels on CD4+CD25highFoxp3+ T cells from patients (a) and healthy controls (b) were determined and correlated with age. A negative correlation was observed for both analyses.

The included patients and healthy controls signed an informed consent and completed a questionnaire concerning personal data, physical examination and BCG vaccination. Blood samples of age-matched healthy controls (n=27) and patients with clinical signs of leprosy (n=10) were collected at the Getúlio Vargas Hospital, Teresina, Brazil. Following diagnosis, patients received standard therapy, and blood samples were collected at the following three time points: (i) when leprosy diagnosis was confirmed and at three (ii) and six (iii) months after initiation of the classical therapy regime. The healthy controls had blood samples collected once. The study protocol was approved by the Federal University of Piauí's institutional review board (CEP-0160.0.045.000-10) and by the Brazilian National Ethics Committee (CONEP), case number 530.719 (CAAE 01006912.2.0000.5214).

T cell purification and plasma isolation

Venous blood samples were collected from patients and controls in EDTA-containing tubes. The blood was used as a source for both Peripheral Blood Mononuclear Cell (PBMCs) and plasma acquisition. PBMCs were recovered from the inter phase of Ficoll gradients according to standard protocols (Amersham Bioscience, Sweden). The cells were subjected to negative MACS selection using a Pan T cell Isolation Kit II (Miltenyi Biotec, BergischGladbach, Germany) to obtain flow-through purified T cells. The plasma samples were separated by centrifugation and stored at -80°C. All plasma samples from an individual patient were assayed simultaneously.

T lymphocyte immunophenotyping by flow cytometry

For flow cytometric analysis, 5x105 T lymphocytes were incubated in the dark at 4°C for 30 min in 25 µl of PBS containing the following fluorescently labeled monoclonal antibodies (Becton-Dickinson, Mountain View, CA, USA): fluorescein isothiocyanate (FITC)-conjugated anti-CD25 and anti-FOXp3; phycoerythrin (PE)-conjugated anti-CD4 and anti-PTK7; peridinin chlorophyll protein (PerCP)-conjugated anti-CD3; and allophycocyanin (APC)-conjugated anti-CD45. Unstained cells were used as negative controls.

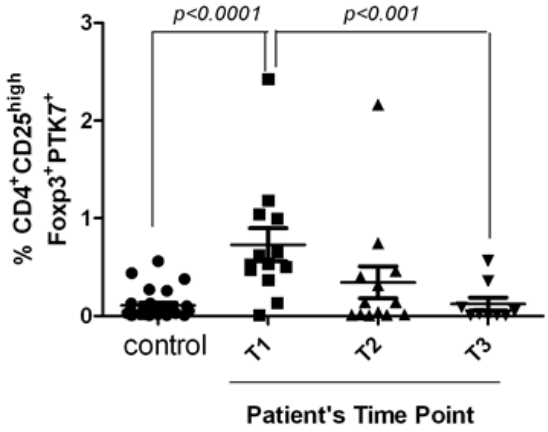

Figure 5: Age-matched patients and controls were compared for PTK7 levels on circulating CD4+CD25highFoxp3+T cells. The patients were sampled in three different times (T1, T2 and T3). The healthy controls were sampled once. Patients at time point one (T1) showed significantly higher PTK7 levels when compared to healthy controls. Moreover, significant differences were found when patients at time points one and three were compared.

The T lymphocytes were analyzed using a FACSCanto II flow cytometer and were gated based on forward scatter (FSC), side scatter (SSC) and surface CD45 expression. The results were analyzed using FACS DIVA software (Becton-Dickinson) and were expressed as the percentage of positive cells.

Cytokine detection

Cytokine levels in the plasma were measured using a Th1/Th2/ Th17 Cytometric Bead Array (CBA) kit (BD Biosciences, San Jose, CA,

USA) according to the manufacturer's instructions. A total of 25 µl of plasma was analyzed for the presence of the following cytokines: IL-2, IL-4, IL-6, IL-10, tumor necrosis factor-alpha (TNF-α), and IL-17. The data acquisition was performed with a FACSCanto II flow cytometer (BD Biosciences), and the results were analyzed using the FCAP program (version 1.0.1).

Statistical Analysis

The data were analyzed using the Kruskal-Wallis test and Dunn's posttest with Prism 4.0 software (GraphPad, Inc., San Diego, CA, USA). All p-values <0.05 were considered significant.

Results

Thymus output is naturally impaired as part of the aging process in humans. Thus, we matched patient and control groups by age, as shown in Figure 1 (age median 40 and 42 years, respectively, p=0.8365) (Figure 1).

We then compared the number of CD4+CD25highFoxp3+ T cells from the control group to the patient group after classical treatment at three different times (times point T1, T2, and T3). We found a significantly higher number of these cells in the patient group at T1 ($p<0.05$) but no difference for either the T2or T3 time point (Figure 2). No differences were observed in the number of CD4+CD25highFoxp3+ T cells when we performed a multi-comparison test amongst the time points T1, T2, and T3 (Figure 2).

Comparison between median values of polar (TT and LL) clinical forms of leprosy revealed that TT patient presented higher numbers of CD4+CD25highFoxp3+ than LL patients (chi square 12.423, data not shown). The possible use of increased CD4+CD25highFoxp3+ T cell number as a tool in the diagnosis of leprosy was evaluated by means of Receiver Operating Characteristic (ROC) curve. The data showed a sensitivity of 71.443% (CI=0.4190 to 0.9161) and specificity of 0.9286% (CI=0.6613 to 0.9982) for a cutoff value 0.5807 (Figure 3).

We then evaluated whether the increased numbers of CD4+CD25highFoxp3+ cells were due to peripheral expansion or augmented thymus output (PTK7+). We found a negative correlation between CD4+CD25highFoxp3+PTK7+ and age in both healthy individuals ($r = -0.7843$; $r2=0.6151$; $p<0.0001$; 95% CI=-0.9084 to -0.5330) and patients ($r = -0.8260$; $r2=0.6823$; $p<0.0005$; 95% CI= -09463 to -0.5047) (Figure 4).

Our results showed that at disease onset (T1), the patients have a higher number of CD4+CD25highFoxp3+PTK7+ than both controls ($p<0.05$) and themselves at the end the treatment ($p<0.05$) (Figure 5).

The use of increased CD4+CD25highFoxp3+PTK7+ T cell numbers as a tool in treatment follow-up of leprosy patients was evaluated with an ROC curve. The results showed a sensitivity of 84.62% (CI=0.5455 to 0.9808) and a specificity of 0.8148% (CI=0.6192 to 0.9370). The cutoff value was 0.1975 (Figure 6).

Discussion

The present study represents the first investigation of PTK7 levels on circulating CD4+CD25highFoxp3+ T lymphocytes in patients carrying an infectious disease.

The significantly higher number of circulating CD4+CD25highFoxp3+ T cells in leprosy patients (in time point T1) compared to age-matched healthy controls ($p<0.05$) suggests the onset of infection causes perturbations within the T cell compartment. There are two non-mutually exclusive hypotheses for explaining the observed T cell compartment perturbation. The first is that we observed a relative and not an absolute increase in the number of Tregs. This possibility is in agreement with findings of Morgun and coworkers [18] that showed a decrease in activated T cells in the blood of patients with heart transplants during rejection. The second hypothesis for the augmented number of Tregs in leprosy is an increase in thymus output.

Although the precise mechanism causing selective output of Treg cells from the thymus is not clear, it has been suggested that feedback mechanisms ensure each lymphocyte that leaves the blood and enters the tissues is replaced by an RTE [19].

As a result, CD4+CD25highFoxp3+ T cells of patients at time point T1 presented significantly higher levels of PTK7 than age-matched healthy controls. Time points T2 and T3 showed no difference regarding the number of circulating Treg cells. This could be explained, at least in part by the triggering of apoptosis as mechanisms of self-limitation for Tregs, as can be inferred from the report of Quaresma and colleagues [20] Since the size of the patients group was limited in this study we were not able to compare the number of Tregs relative to the age between the polar clinical forms of leprosy, although we found a higher number of CD4+CD25highFoxp3+ cells in patients with TT clinical form. This is an interesting find that, at glance, seems to contrast to that one reported by Quaresma and colleagues [21] about different clinical forms of leprosy and cytokine production. However, while we worked with circulating T lymphocytes, Quaresma an colleagues worked with infiltrating lymphocytes.

From the diagnostic point of view, the sensitivity and specificity of PTK7 levels on circulating CD4+CD25highFoxp3+ could be used as an auxiliary tool for the diagnosis of leprosy. It may also be a useful tool for disease monitoring throughout treatment. Thus, we believe that the assessment of CD4+CD25highFoxp3+PTK7+ cells in the blood represents a promising approach to monitoring leprosy evolution following treatment and warrants future investigation.

It is important to note that the number of CD4+CD25highFoxp3+ PTK7+cells decreased continuously from time point T1 (where we found statistical significance) to time point T3 in patients. Additionally, there were no differences observed between T3 and healthy controls. This result suggests that the inflammatory process observed at the onset of disease augments circulating Treg numbers. The increase in the number of Treg cells is thought to be necessary because a failure to control a strong immunological response toward pathogenic organisms can lead to considerable tissue damage at the site of infection and causes deleterious pathology. Consistent with this possibility, injection of regulatory T cells can prevent SCID mice from developing severe pneumonitis (caused by Pneumocystis carinii) following reconstitution of animals with CD4+ CD25- T cells [22].

The infiltration of Treg cells at an inflammatory site [23] may recruit more Treg cells to that inflammatory site [24]. Thus, Tregs appear to attract Tregs. We found no quantitative difference in the blood levels of cytokines between patients and healthy controls that explains the higher number of Treg cells in leprosy patients. However, it is possible that soluble factors such as CCL1 are involved [24].

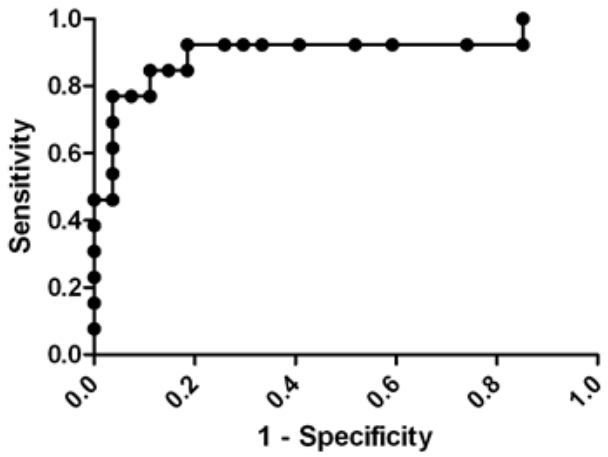

Figure 6: PTK7 expression on circulating CD4+CD25highFoxp3+T is a useful parameter for leprosy treatment follow- up based on the ROC curve.

Conclusion

In conclusion, we provided evidence that augmented blood levels of CD4+CD25highFoxp3+ T cells at the onset of leprosy are most likely due to de novo output from the thymus. Moreover, measuring CD4+CD25highFoxp3+PtK7+ in blood could be an auxiliary tool in monitoring patient treatments.

Acknowledgements

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