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Regulatory T Cells (Treg) Participate in the Development of Anergy in Murine Leprosy

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ABSTRACT

Specific loss of cell-mediated immunity to *Mycobacterium leprae*, is a characteristic feature of human lepromatous leprosy. This phenomenon is called cellular anergy and it is considered the reason for the disease progression. It is not clear the reason for anergy in human leprosy, but several experimental-based explanations have been proposed. One such explanation is the excess of suppression exerted by regulatory T cells (Treg). In murine leprosy, caused by *Mycobacterium lepraemurium*, cellular anergy is also observed and the model offers the opportunity to further explore the phenomenon vertically, in the same animal because of the availability of syngeneic strains, and during the whole time of infection. Syngeneic Balb/c mice were inoculated with MLM, and the evolution of the infection was monitored over 4 months based on diverse parameters: body weight, splenomegaly and hepatomegaly, presence of bacilli, and presence of CD4+CD25+Tbet and CD4+CD25+FoxP3 cells. It was found, by flow cell cytometry, that the first immune response in the infected animals reflected the activity of Th1 (Tbet+) cells and this response was then substituted by a predominant T-FoxP3 response, thus suggesting that CD4+CD25+FoxP3 cells play a role in the development of anergy in the malignant form of murine leprosy.

Keywords: Anergy; Murine Leprosy; Regulatory T cells (Treg)

Introduction

One of the most intriguing features of leprosy is the development of cellular anergy specific to the leprosy bacillus (*Mycobacterium leprae*). Anergy develops in lepromatous leprosy, the malignant form of the disease, and is responsible for the lack of the body's immune defense to leprosy. Faults in diverse mechanisms of cell-mediated immunity have been described since long ago [1] and presently several of these mechanisms have been confirmed but the information remains disperse [2,3]. Whether anergy is due to intrinsic (genetic) traits of the host or is provoked by the bacillus itself is still an unanswered query, however, considering the new observations, it seems that peculiarities of *M. leprae* and intrinsic host-susceptibility play a synergistic role in the development of anergy. Analyzing the evolution of chronic diseases, such as leprosy, in human beings is a difficult task because the studies have to be done in a transversal manner and cannot be done vertically due to the disease›s chronicity that may span for many years, hence, following the progression of the disease in the same individual is almost impossible; here, animal models are of utmost importance, however, for leprosy, nine-banded armadillos (Dasypus novemcinctus) and non-human primates (chimpanzees and mangabey monkeys), are the only experimental models for leprosy, both of them expensive, and the latter one, subjected to strict ethical restrictions [4].

There is, however, a disease in the mouse (murine leprosy) that shares several characteristics with human leprosy, though they are not the same disease [5]. One of the shared features is anergy and this has allowed us to investigate the cellular mechanisms of anergy along the development and progression of the disease in the same individual. For an efficient immune response to occur, a well-balanced network of interactions between cells and molecules must exist. [6,7] In a simple form, microorganisms, either infectious or non-infectious, are captured by phagocytic cells, macrophages and dendritic cells, which inactivate, kill, and degrade them to present the resulting antigenic peptides to naive T cells which then differentiate into diverse T cell sub-populations (Th1, Th2, Th17, Th9, Tfh, Treg, etc.) with a variety of functions. Th1 cells, through the production of proinflammatory cytokines, are the master effector cells of cellular immunity whose activity is mainly controlled by the regulatory T cells (Treg). [8,9]. Th1 cells produce gamma-interferon (IFNγ) and Tumor Necrosing Factor (TNFβ) which are potent macrophage activators, and the activated macrophages are enabled to efficiently destroy microorganisms, including *M. leprae* and *M. lepraemurium*. In the absence of Th1-cell activity (anergy), macrophages do not get activated and lower their bactericidal activity. Inactivation of Th1 cells may occur because of the bacteria-driven regulation of regulatory Treg cells. In this communication, we present evidence that suggests that anergy in murine leprosy occurs, at least in part, due to the up regulation of Treg cells activity that surpasses the activity of Th1 cells, blocking in this manner their macrophage activating capacity. Without Th1 cells stimulation, macrophages rest in a passive status, unable to get rid of the microorganisms. This has also been hypothesized to occur in human leprosy with macrophages forced to acquire the M2 (anti-inflammatory) phenotype [10].

Material and Methods

Mice

Female, Balb/c mice, 8 weeks old, were used in the study. The animals were handled in accordance with the national norms for the use of laboratory mice (NOM-062-ZOO-1999; NOM-029-ZOO-1995 and NOM-033-ZOO-1995).

Bacilli

Mycobacterium lepraemurium (MLM) was isolated from the spleen of mice undergoing a 4-monts infection with MLM. Bacilli were isolated by using the method described in [11].

Infection

Forty mice were intraperitoneally inoculated with 20 x 106 bacilli and the infection was left to proceed for 17 weeks. Every 2 weeks, four mice were sacrificed by CO2 inhalation and their spleens and livers were removed. Spleens were used for cell collection, and bacilloscopic studies; livers were used to monitor the progression of the disease.

Flow Cytometry

Spleen cell suspensions were prepared in a standard manner, counted, and adjusted to 10x106 cells per ml. Four, one million splenocyte aliquots, were separately incubated with antibodies to mouse CD4 (FITC) and CD25 (APC) and, after fixation and permeabilization, they were treated with anti-mouse FoxP3 (PE), T-bet (PeCy7) and rat IgG2b isotype (PE), all of them from Biolegend, San Diego, CA, USA. Autofluorescence, compensation and isotype controls were all considered in the assay. Washed, stained cell suspensions were suspended in paraformaldehyde, acquired in a BD FACS Aria I cytometer coupled to the FACS Diva software. Finally, the results were analyzed with Flow Jo software (See methodological details in https://www.cellsignal.com/learn-and-support/protocols/protocol-12653-12632-flow?).

Tissue Sections

Small pieces of the spleen and liver were fixed in 4% formalin for 72 hours and then processed for paraffin embedding and sectioning in a Leica microtome (Leica Biosystems, IL, USA). Four-micron thick tissue sections were deparaffinated, hydrated, and stained with phenol-fuchsin for acid-fast bacilli (Ziehl-Neelsen stain) and hematoxylin-eosin for general histology.

Cell Activation

Five thousand splenocytes of each mouse were incubated in 100 µl of complete DMEM medium (10% calf fetal serum, penicillin, 100 U/ml; gentamicin, 50 µg/ml, and essential and non-essential amino acids) containing nil, 500 ng of concanavalin A, or 5 µg of a whole soluble extract of *M. lepraemurium* prepared by sonication, centrifugation, and filtration through 0.22 µm membranes. Protein content was measured using Lowry's method and bovine serum albumin as a standard. Cell cultures, run in quadruplicate in 96-well microplates, were incubated for 5 days at 37°C, 5% CO2 in a humid atmosphere. Cell proliferation was assessed by counting and measuring the number of cell clones developed under each time condition. ImageJ V 1.8.0 software (public domain) was used for the measurements.

Results

The liver and spleen are target organs in murine leprosy, as are lymph nodes, skin, and bone marrow. Due to its non-lymphoid nature, the liver is appropriate for studying the origin and development of granulomas in murine leprosy as well as the subjacent immunologic mechanisms involved in their maturation. Spleen, on the other hand, being a lymphoid organ allows for the study of the characteristics, functions, and interactions of the lymphoid immune cells (lymphocytes, macrophages, and dendritic cells). Because of the inflammatory reaction provoked by the infection, these two organs increase in volume and their hyperplasia becomes an index of the disease.

Bacilli

In the present four-month study, bacilli appeared in these organs within 5 weeks of infection and from here the infection progressed to the end of the study at 120 days (Figure 1 & 2). Comparable results were found in the liver (images not shown).

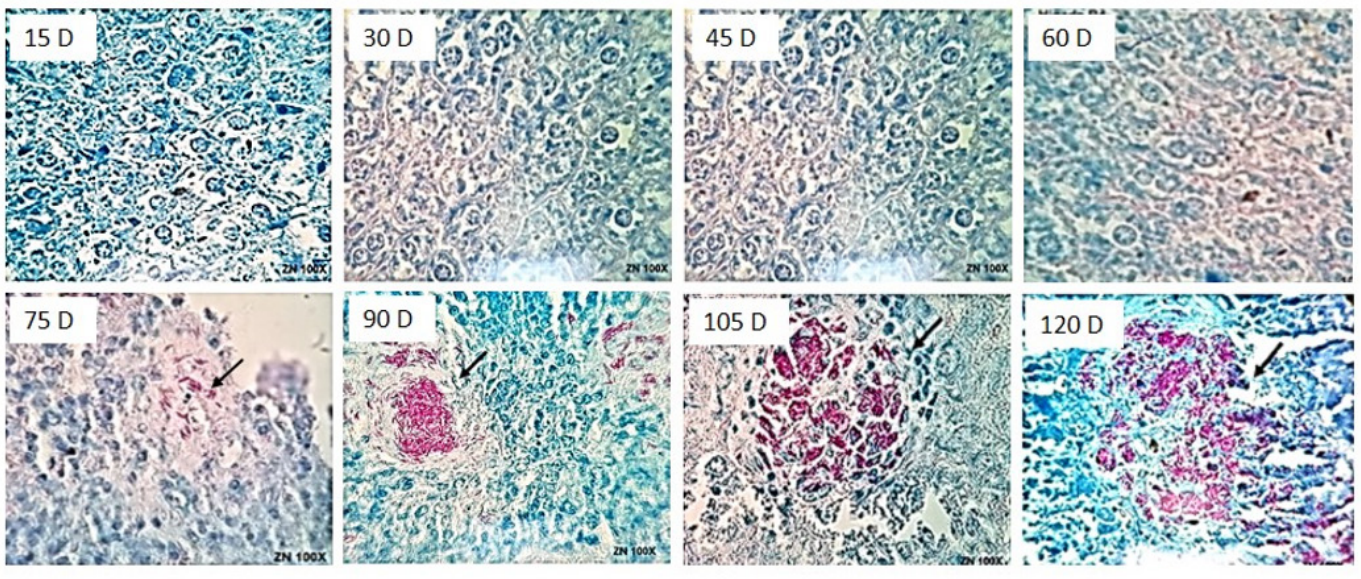

Figure 1: Multiplication of *M. lepraemurium* in the spleen lesions of mice infected with the microorganism. Notice the absence of bacilli during the first two months of infection and the multiplication of bacilli starting at 75 days. We hypothesize that effector Th1 Tbet+ cells were acting within the first 60 days of infection, and Treg FoxP3+ cells started acting from day 75 to the end of the experiment at 120 days. Hematoxylin-Eosin and Ziehl-Neelsen stains. 100X.

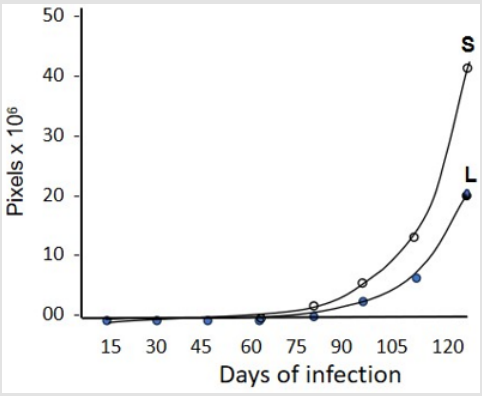

Figure 2: Number of bacilli (in pixels) in spleen (S) and liver (L) along the infection time. Notice that bacilli started to appear on the third month of infection. Each point is the average number of bacilli found in the 4 mice of the corresponding group, standard errors are omitted to simplify the graph. Ziehl-Neelsen stain, 100X.

Flow Cytometry

Flow cytometry was used to detect the expression of CD4+CD25+ cells carrying the transcription factors Tbet (Th1) and FoxP3 (Treg) cells. The strategy to quantify the numbers of CD4+CD25+ carrying the transcription factors Tbet (Th1) and FoxP3 (Treg) is depicted in Figure 3. This figure corresponds to the analysis of splenocytes from a normal mouse, but the strategy was followed for the analysis of the four mice in each group of infection (15 days, 30 days, 45 days, 60 days, 75 days, 90 days, 105 days and 120 days), From the individual data, the graph in Figure 4 was constructed. It is observed that the expression of T cells carrying the transcription factor Tbet (Th1 cells) appeared early, within the first two months of infection, whereas the cells carrying the transcription factor FoxP3 (Treg) started to appear at the beginning of the third month of infection.

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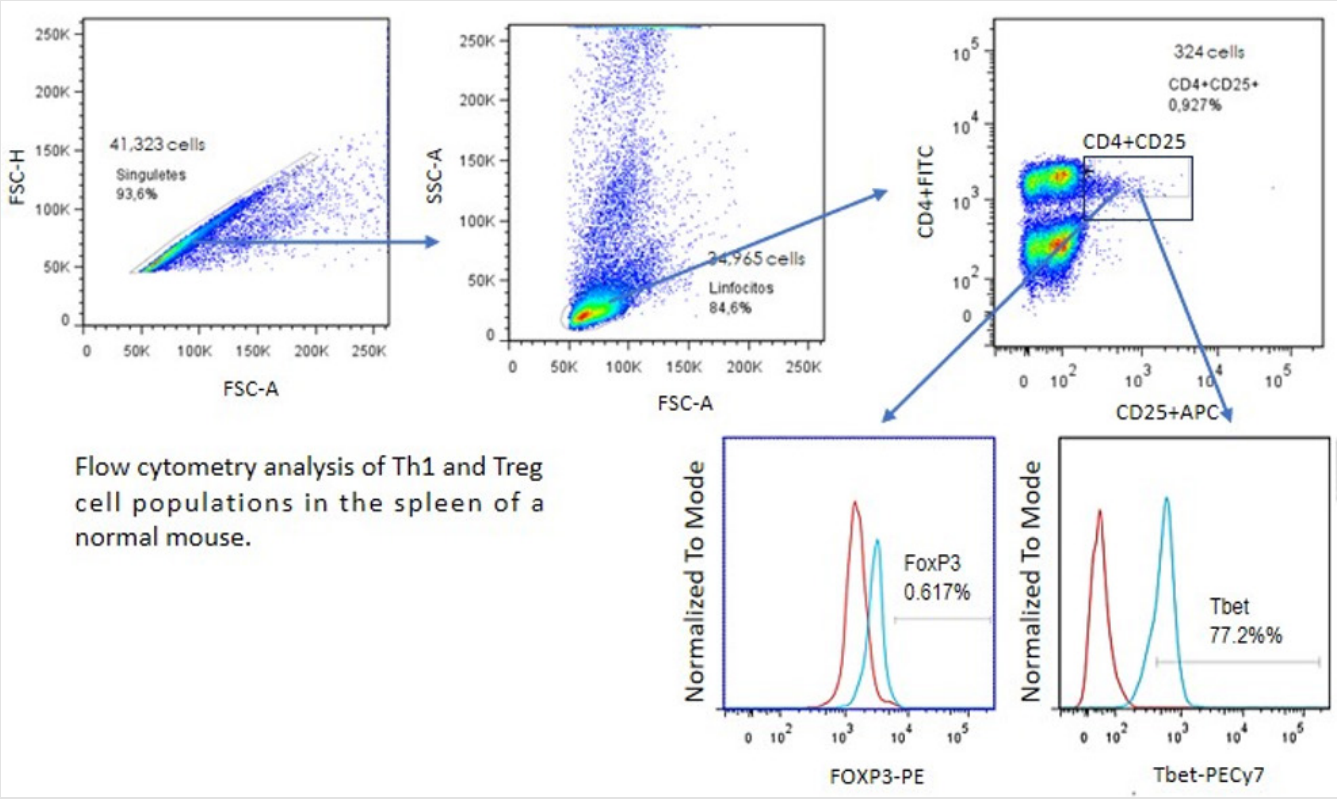

Figure 3: The figure illustrates the strategy followed for the quantitation of CD4+CD25+FoxP3+ (Treg) and CD4+CD25+Tbet+ (Th1) cells. In the histograms, the red peak corresponds to the isotype and the blue peaks correspond to Treg FoxP3 cells (left panel) and Th1 Tbet cells (right panel). This strategy of analysis was applied to the study of the splenocytes of mice inoculated with *M. lepraemurium* for up to 120 days.

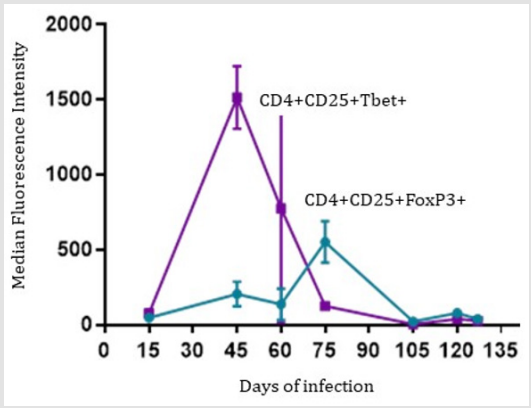

Figure 4: Sequential expression of Tbet (Th1) and FoxP3 (Treg) cells along the infection time of mice with *M. lepraemurium*. Notice the predominant activity of Th1 cells during the first two months of infection, and the predominant activity of Treg cells later.

Cell Proliferation

Splenocytes from the infected mice stimulated with Con-A proliferated stronger at the beginning of the infection than at the more advanced disease but the proliferative response was always significant. Splenocytes from the infected mice stimulated with the extract of *M. lepraemurium* did not proliferate at any time of the infection. Splenocytes incubated in the presence of plain culture medium (DMEM) showed minimal basal proliferation. These data are depicted in Figures 5 & 6 (graph).

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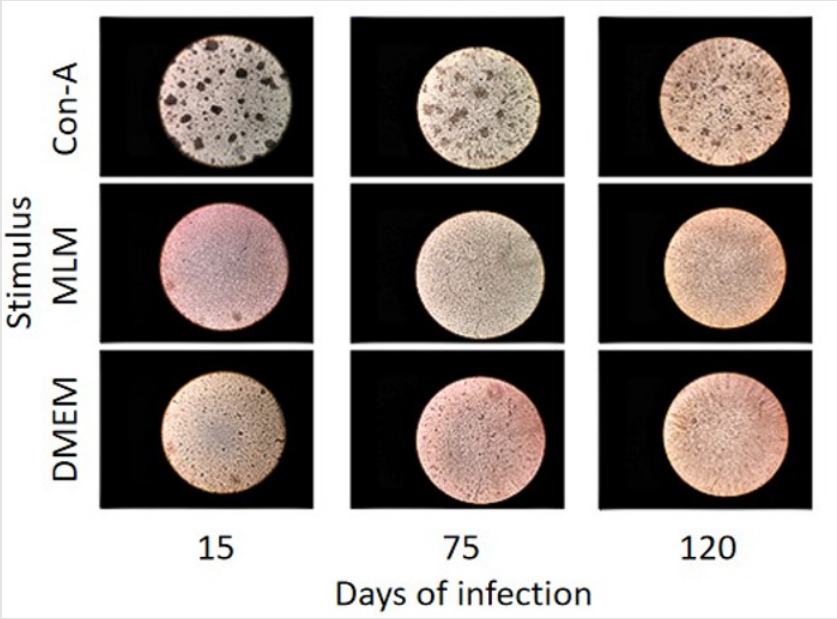

Figure 5: Proliferation of splenocytes of mice suffering from progressive infection with *M. lepraemurium*. Cells were stimulated with concanavalin A (upper row), soluble extract of *M. lepraemurium* (middle row), or plain DMEM (lower row). The infection caused a progressive loss of the response to Con-A while MLM extract did not stimulate the cells at any time studied. MLM seems to be an anergy-induced microorganism.

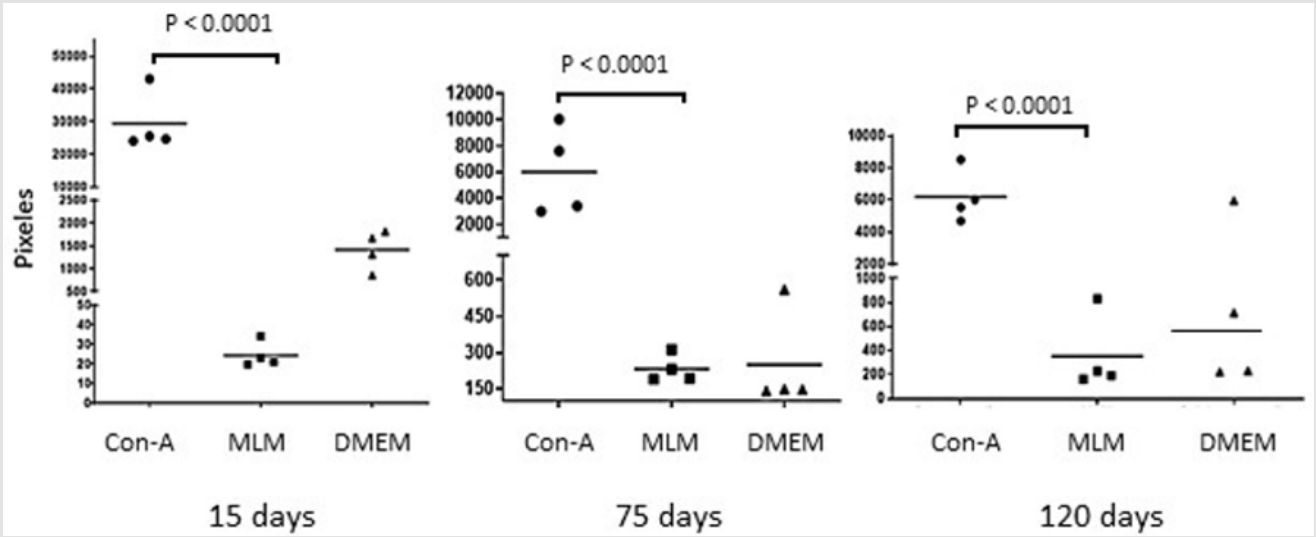

Figure 6: Proliferative response to Con-A, MLM, and DMEM of the splenocytes of mice infected with *M. lepraemurium* along 120 days of infection. Notice the significant proliferative response to ConA and the lack of proliferative response to the extract of MLM at all times of infection. One way ANOVA with post hoc Tukey test.

Discussion

Anergy in human lepromatous leprosy, the "malignant" form of the disease, has been documented in several reports over many years. This condition does not exist in tuberculoid leprosy, the "benign" form of the disease. Without treatment, patients with lepromatous leprosy are unable to control the proliferation and dissemination of *M. leprae*; on the contrary, patients with tuberculoid leprosy can control the disease even, in some cases, without treatment. However, both forms of leprosy respond positively to the anti-leprosy treatment regimen recommended by the WHO (dapsone, rifampicin and clofazimine). Malignancy in lepromatous leprosy obeys, at least in part, to the development of anergy in these patients. Anergy in leprosy has been linked to the emergency of regulatory T cell activity. [12-14] Anergy nullify the immunologic capacity of the patient to cope with the bacillus. Although there is a lot of information on the mechanisms of immunity in leprosy, anergy is still a phenomenon poorly understood (Rojas-Espinosa [1]) Murine leprosy, is a disease that traverse all forms of leprosy from the tuberculoid-like to the lepromatous-like disease (Rojas-Espinosa [5]) Murine leprosy is an excellent animal model for studying the development of anergy that appears in the middle stages of the infection.

In the present study we have given evidence on the participation of regulatory T cells (Treg) in the development of anergy. While the activity of Th1 (CD4+CD25+Tbet+) is in function, the disease stays under control. When the activity of Th1 cells is down-regulated due to the activation of Treg (CD4+CD25FoxP3+) cells, the disease becomes uncontrolled and progresses to the death of the animals. Down regulation of Th1 cells by Treg cells occurs through the release of IL-10 and IL-4 by Treg cells. Despite the evidence given in this paper on the sequential activation of Treg cells, as a cause of anergy in murine leprosy, the molecular or cellular mechanisms of their activation remain to be elucidated. Anergy in murine leprosy is a situation that can be variably reversed using immunomodulators such as dialyzable leukocyte extracts, omega 3 fatty acids, and sodium butyrate. [15,16] In the present study, the diminution in the number of Treg cells in the late states of the infection correlated with the decrease in the absolute numbers of T cells in the advanced stages of the disease (Figure 7).

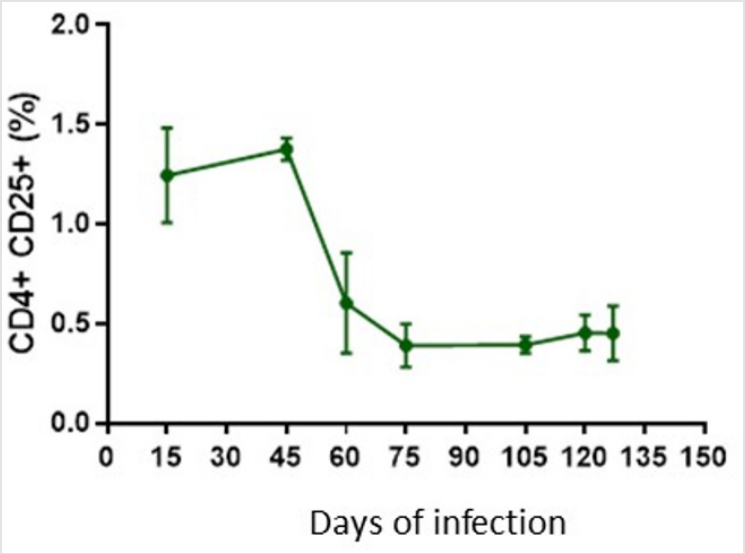

Figure 7: Reduction in the number of CD4+CD25+ T cells in the spleen of mice suffering from leprosy. In the present study a marked drop in the number of T cells, including Th1 and Treg cells, occurred on day 60 of the infection and was kept low until the end of the study at 130 days.

Conclusion

Anergy in leprosy (and in murine leprosy) is a condition that accompanies and worsens the malignant (lepromatous) form of the disease. Several mechanisms for the anergy have been proposed and the conceptualization that we have on the phenomenon is that it is a multi-factorial complication of the disease. Lack of *M. leprae*-reactive lymphocytes, activation of suppressor lymphocytes and macrophages, immunologic synapse interference (downregulation of coestimulatory molecules), shift of macrophages from the M1 (proinflammatory) phenotype to the M2 (anti-inflammatory) phenotype, and *M. leprae*-driven upregulation of regulatory T cells, are some of the possible causes of anergy in human [1] and murine [5] leprosy. In the present study we have given evidence of the participation of Treg cells in the anergy in murine leprosy. Neutralization of Treg cells activity through the use of blocking antibodies to IL-10, TGFβ, and IL-4, might improve the outcome of the disease.

Conflicts of Interest

The authors declare no conflicts of interest.

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Activities

OR-E designed, supervised the study, and wrote the manuscript; T V-M and O R-C performed the flow cytometry analysis; P A-P and E B-V ran the experiments.

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