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Expression of FoxP3 in different forms of leprosy and reactions

Expressão de FoxP3 em diferentes formas de hanseníase e reações hansênicas

Chalita Patricia Lima; Elisa M. Costa; Lucas F. Sampaio

Universidade Estadual de Goiás (UEG), Anápolis, Goiás, Brazil.

ABSTRACT

Although regulatory T cells (Treg) are important in the immune regulation of various pathological processes, the role of these cells in the immunology of leprosy still needs to be better evaluated. The objective of this work is to quantify the FoxP3, the main marker of Treg cells, in the different forms of leprosy, with and without leprosy reaction. Expression of Treg cells was investigated using the enzyme-linked immunosorbent assay (ELISA) method to measure the FoxP3 marker in unstimulated plasma as well as in the peripheral blood mononuclear cells (PBMC) supernatant after incubation with *M. leprae* sonicated antigen (MLSA) for 72 hours. After stimulation with MLSA, a strong increase in FoxP3 expression was observed in multibacillary leprosy (MB), type 1 leprosy reaction (T1R) and type 2 leprosy reaction (T2R) patients. However, the group that presented the highest expression of FoxP3 was T1R, about six times more than that of healthy individuals and twice as many as T2R patients. Thus, this study suggests that Treg plays an important role in the etiopathogenesis of leprosy reactions, regulating the exacerbated acute inflammatory process, avoiding severe and incapacitating lesions. This study also demonstrates that ELISA, a simple and affordable technology, can be used in routine measurement of the FoxP3 marker.

Key words: *Mycobacterium leprae*; erythema nodosum; Treg.

RESUMO

Embora as células T reguladoras (Treg) sejam importantes na regulação imunológica de vários processos patológicos, o papel delas na imunologia da hanseníase ainda precisa ser mais bem avaliado. O objetivo deste trabalho é quantificar o nuclear transcription factor P3 *(FoxP3), principal marcador de células Treg, nas diferentes formas de hanseníase, com e sem reação hansênica. A expressão de células Treg foi investigada utilizando o método de ensaio de imunoabsorção enzimática (ELISA) para medir o marcador FoxP3 em plasma não estimulado, assim como no sobrenadante de células mononucleares do sangue periférico (PBMC) após incubação com antígeno sonicado de* M. leprae *(MLSA) durante 72 horas. Após estimulação com MLSA, um forte aumento da expressão de FoxP3 foi observado em pacientes com lepra multibacilar (LM) com reação do tipo 1 e 2. No entanto, o grupo que apresentou maior expressão de FoxP3 foi o de pacientes com reação do tipo 1, com cerca de seis vezes mais pacientes do que o de indivíduos saudáveis e duas vezes mais que os com reação do tipo 2. Portanto, este estudo sugere que o Treg desempenha um papel importante na etiopatogenia das reações hansênicas, regulando o processo inflamatório agudo exacerbado e evitando lesões graves e incapacitantes. Este estudo também demonstra que o ELISA, uma tecnologia simples e acessível, pode ser usado na medição de rotina do marcador FoxP3.*

Unitermos: Mycobacterium leprae*; eritema nodoso; Treg.*

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RESUMEN

Aunque las células T reguladoras sean importantes en la regulación inmunológica de varios procesos patológicos, su papel en la inmunología de la enfermedad de Hansen, o lepra, todavía necesita ser correctamente evaluado. El objetivo de este trabajo es cuantificar el factor de transcripción nuclear P3 (FoxP3), principal marcador de células Treg, en las diferentes formas de lepra, con y sin reacción leprosa. La expresión de células Treg fue investigada con el ensayo por inmunoabsorción ligado a enzimas (ELISA) para medición del marcador FoxP3 en plasma no estimulado así como en el sobrenadante de células mononucleares de sangre periférica (PBMC) luego de incubación con antígeno sonicado de M. leprae *(MLSA) por 72 horas. Después de estimulación con MLSA, un fuerte aumento en la expresión de FoxP3 fue observado en pacientes con lepra multibacilar (MB) con reacción del tipo 1 y 2. Sin embargo, el grupo que presentó mayor expresión de FoxP3 fue el grupo de pacientes con reacción tipo 1, con alrededor de seis veces más pacientes que el de sujetos sanos y dos veces más que con los de reacción tipo 2. Por consiguiente, este estudio sugiere que Treg desempeña un papel importante en la etiopatogenia de las reacciones leprosas, regulando el proceso inflamatorio agudo agravado y evitando daños graves e incapacitantes. Este estudio también demuestra que ELISA, una técnica simple y accesible, puede ser usada en la medición de rutina del marcador FoxP3.*

Palabras clave: Mycobacterium leprae*; eritema nudoso; Treg.*

INTRODUCTION

Regulatory T cells (Tregs) are a subset of cells characterized by the nuclear transcription factor P3 (forkhead box P3 or FoxP3)[1,2]. Although Tregs play an important role in most infectious diseases, their role in the immunopathology of leprosy has not yet been elucidated. Some studies have shown an increase in the number of Treg cells in leprosy paucibacillary (PB) form[3,4], whereas other researchers have observed high levels of Treg in the multibacillary (MB) forms[5,6]. In relation to studies with leprosy reactions, researchers observed a statistically significant increase in FoxP3 expression in patients with type 1 leprosy reaction (T1R) when compared to type 2 leprosy reaction (T2R)[7,8]. In contrast, other researchers found a greater increase in FoxP3 expression in T2R[3]. Thus, in this study we investigated the frequency of FoxP3, a marker of Treg cells, in different forms of leprosy and leprosy reactions. We also investigated the possibility of using the enzyme-linked immunosorbent assay (ELISA) technique, a simple, inexpensive and quite accessible assay in the measurement of FoxP3 marker.

METHODS

For quantification of the FoxP3 marker in the peripheral blood, five groups were recruited. Groups 1 and 2 consisted of leprosy patients PB and MB respectively, without reaction, who had been newly diagnosed and who had never undergone multidrug therapy. Groups 3 and 4 had patients with T1R and T2R, respectively. Samples from all patients with reaction were collected at the beginning of the reaction episode, prior to the use of corticosteroids or thalidomide. Patients were assigned to leprosy groups based upon their immediate diagnosis, and were subsequently thoroughly characterized according to Ridley-Jopling criteria, taking into account clinical, bacilloscopic and histopathological findings. For group 5, healthy endemic controls (HEC), individuals without clinical signs or family history of leprosy or tuberculosis were recruited. All groups were composed of 10 individuals. Recruitment of leprosy patients, with or without reaction, was performed in six Basic Health Units in Goiânia, Brazil. The blood sample collection of all the patients occurred between the months of January and September, 2017. This study was approved by the Ethics Committee of Hospital das Clínicas de Goiânia (protocol no. 12962).

The plasma FoxP3 concentration (in the absence of stimulation) and in the peripheral blood mononuclear cell cultures (PBMC) supernatants, stimulated with the *M. leprae* sonicated antigen (MLSA), was determined by ELISA with the human FoxP3 kit, according to the manufacturer's instructions (LifeSpan BioSciences – Seattle). Cultures with PBMC were prepared according to protocol set forth in Chaduvula *et al*. (2012)[9]. The statistical analysis was performed with Graphpad Prism v.5. Mann-Whitney test was used for comparison between two groups of patients.

RESULTS AND DISCUSSION

The expression of the FoxP3 marker was investigated using the ELISA, firstly in the plasma without stimulation and, subsequently, in the supernatant of PBMCs stimulated with *M. leprae* cell sonicate (MLCS) antigen. The results show that there was no difference in FoxP3 expression measured directly in the plasma, in the absence of stimuli among the groups analyzed.

After stimulation with the MLCS antigen, PBMCs of all groups demonstrated a statistically significant increase in FoxP3 expression when compared to healthy individuals (**Figure**). The median of FoxP3 in the MB group was 16.06 ng/ml. This value is significantly higher than in the HEC and PB groups, as shown in the Figure. Although this study has used a method different from previous studies, our work is in agreement with most of the studies that have already researched the Tregs in leprosy, demonstrating a larger number of FoxP3+ cells in MB patients than in PB ones[10-13]. We believe that this increase is related to the T cell anergy observed in the MB form of the disease. FoxP3+ cells would then have a crucial pathogenic role in these MB individuals, since the elevation of these cells could increase the suppression of Th1 response, reducing the release of interferon gamma (IFN-γ) and other Th1 cytokines, which would favor the survival and proliferation of the bacillus. The results of this study are divergent from a previous study showing more FoxP3 cells in PB patients when compared to MB patients[3]. We believe that the difference is related to the fact that Attia *et al*. (2010)[3] investigated circulating FoxP3+ cells, but did not culture them, as was done in our study.

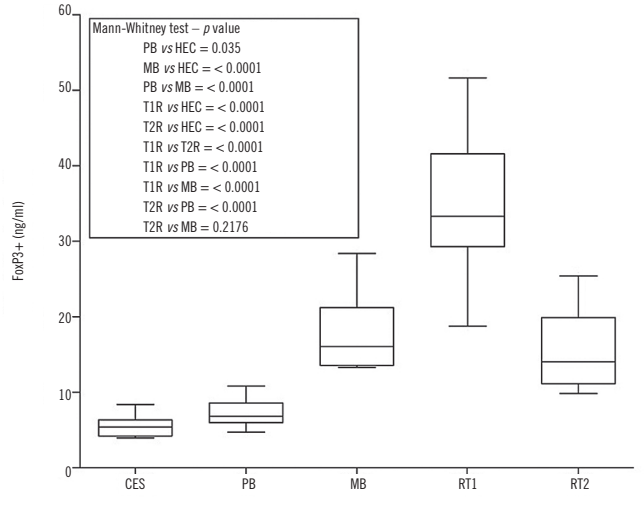

FIGURE – *Quantification by ELISA of FoxP3 marker in PBMCs of patients and healthy individuals undergoing challenge*

PBMC from leprosy patients and controls were stimulated in vitro *with MLCS. The total number of FoxP3 Treg cells was determined by ELISA. The results are presented as box plot, interquartile (25%-75%) and black line within each box indicates the median value;* n = *10 per group.*

PBMC: peripheral blood mononuclear cells; MLCS: M. leprae *cell sonicate; FoxP3: nuclear transcription factor P3; Treg: regulatory T cells; ELISA: enzyme-linked immunosorbent assay; HEC: healthy endemic controls; PB: paucibacillary leprosy; MB: multibacillary leprosy; T1R: type 1 reaction; T2R: type 2 reaction.*

The group with the highest amount of FoxP3 was T1R. The amounts of FoxP3 in T1R patients were about six times higher than in the PB group and almost double the amount observed in MB and T2R patients (Figure). This same trend of higher amounts of Treg cells in T1R was also observed in previous studies, which showed more FoxP3 cells in R1T patients both *in situ* and in circulating blood cell culture[4, 8, 14]. A recent study of 2018, also performed with leprosy patients from Goiânia, showed in situ that people with leprosy type 1 has more FoxP3 + cells than T2R patients. According to Costa *et al*. (2018)[14], the small amount FoxP3+ cell during T2R, in comparison to T1R, is compatible with the extensive and somehow uncontrolled clinical manifestations associated with T2R. Under the circumstances of decreased Treg cells expression, greater tissue and nerve damage might occur[15]. On the other hand, our results of increased FoxP3+ cells during T1R suggest a possible suppressive Treg to control the cell-mediated immunity exacerbated in T1R. That suppression is beneficial. In other words, the increase of Tregs during the T1R seems to be related to the reduction of the neural damage that can occur in this type of reaction. Although it was not the intention of the present study, we verified that after the end of the treatment only one of the ten R1T patients presented neuritis and physical disabilities related to the reaction. On the other hand, six of the ten R2T patients had neuritis and/or physical disabilities related to leprosy.

The balance between Treg and Th17 cells seems to be directly related to the manifestation of leprosy type 1 or 2 reactions. Previous studies have shown that T2R patients have more Th17 cells than T1R patients. However, T1R patients have more Tregs[12, 14, 15]. Although we did not evaluate Th17 cells in our study, we believe that T2R patients would present more of that subpopulation of cells than patients with reverse reaction, what would also help explain why type 2 reaction is generally more severe and harmful to peripheral nerves than T1R, since high amounts of Th17 may be deleterious to the tissue itself. We believe that in a near future it will be possible to control leprosy reactions by regulating Treg and Th17 cells.

In the present study, we measured the FoxP3 marker by ELISA in circulating cells, while most studies investigated FoxP3 in leprosy using more sophisticated assays, such as flow cytometry[3, 5, 6, 10, 15]. The flow cytometry method is a costly technique, not very accessible and available in very few laboratories in Brazil. However, the results of the present study demonstrate the efficacy in measuring FoxP3 marker by ELISA tests, which is a simple and much cheaper assay, being able to replace the more complex and expensive tests.

The major limitation of this study was the non-evaluation of other Treg markers, such as CD25 and the cytokine transforming

growth factor beta (TGF-β), proving that the measured FoxP3+ cells were actually regulatory T cells. Studies with a larger number of patients and controls, using other markers, should be performed for a more detailed analysis of Treg in leprosy. Another limitation of this study was not to compare ELISA technique with other methods for the measurement of FoxP3. Therefore, we believe that further studies comparing the ELISA technique directly with flow cytometry and immunohistochemistry should be performed to confirm the potential use and efficacy of the ELISA in the measurement of FoxP3 + cells.

CONCLUSION

The data presented here provide important information about the involvement of FoxP3 cells in the different forms of leprosy and leprosy reactions, demonstrating the relevant role of these cells in the immunopathology of the disease. Our results suggest that the increased number of FoxP3+ cells in T1R patients could be beneficial to the host as a protection mechanism in an attempt to regulate a possible exacerbated immune response against *M. leprae*. That is, FoxP3+ cells would regulate the acute inflammatory process, avoiding a very strong inflammation, which could cause serious and incapacitating lesions on the nerves. The results of this study also suggest that FoxP3+ cells decrease the Th1 immune response in MB patients, which may allow the survival and dispersion of the bacilli in these forms of leprosy. In addition, the study also shows that a simple ELISA assay can be used to measure the FoxP3 marker, as it substitutes more expensive and less accessible techniques, like flow cytometry.

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CORRESPONDING AUTHOR

Lucas Henrique Sampaio 0000-0002-2256-1883
e-mail: lucas.sampaio@ueg.br or lucashfs@gmail.com