Original Article

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The importance of absolute quantification of regulatory T Lymphocytes (TREG) in the course of HIV virus infection

A importância da quantificação absoluta de Linfócitos T Regulatórios (TREG) no curso da infecção pelo vírus HIV

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Resumo

INTRODUÇÃO. Os linfócitos T regulatórios (TReg) são células especializadas na supressão de respostas imunes superreativas como na infecção pelo HIV. OBJETIVOS. Verificar a concentração absoluta de linfócitos TReg durante o curso da infecção pelo HIV em pacientes em diagnóstico ou já em tratamento. MATERIAIS E MÉTODOS. Realizado com 50 indivíduos que procuraram espontaneamente um laboratório particular de Belém - Pará para realizar exames de quantificação de linfócitos T CD4+ e T CD8+ e carga viral (Grupos 2 e 3) ou somente hemograma (Grupo 1 - controle), no período de julho de 2018 a agosto de 2019. O Grupo 2 foi formado por pessoas que realizaram Reação em cadeia da Polimerase da Transcriptase Reversa em Tempo Real (RT-PCR) para HIV1/HIV2 em diagnóstico; e Grupo 3 formado por pessoas que realizaram RT-PCR para HIV1/HIV2 e já estavam em tratamento. RESULTADOS. No Grupo 1 observou-se mediana de idade de 56,5 anos e dependência estatística (p = 0,00451) entre os valores absolutos de Linfócitos T CD4+ e TReg. No Grupo 2 observou-se mediana de idade de 39,5 anos e dependência estatística (p=0,00234) entre valores absolutos de linfócito TReg baixos em pacientes com carga viral detectável alta. No Grupo 3 a mediana de idade foi de 40,5 anos e houve dependência estatística (p = 0,00188) entre os valores absolutos de Linfócitos T CD4+ e de Linfócitos TReg. CONCLUSÃO. Nossos dados sugerem que a terapia antiretroviral aumenta a quantidade de leucócitos totais e linfócitos T CD4+ em função, provamente, do aumento de linfócitos TReg.

Palavras-chave: Linfócitos T Regulatórios, Vírus da Imunodeficiência Humana, HIV, Terapia Antirretroviral de Alta Atividade

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Abstract
INTRODUCTION. Regulatory T lymphocytes (TReg) are cells specialized in suppressing overactive immune responses such as in HIV infection. OBJECTIVES. To verify the absolute concentration of TReg lymphocytes during the course of HIV infection in patients undergoing diagnosis or already undergoing treatment. MATERIALS AND METHODS. Conducted with 50 individuals who spontaneously sought a private laboratory in Belém - Pará to perform tests to quantify CD4+ and T CD8+ lymphocytes and viral load (Groups 2 and 3) or just blood count (Group 1 - control), in the period of July 2018 to August 2019. Group 2 was formed by people who performed Real Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) for HIV1/HIV2 in diagnosis; and Group 3 formed by people who underwent RT-PCR for HIV1/HIV2 and were already undergoing treatment. RESULTS. In Group 1, a median age of 56.5 years and statistical dependence ($p = 0.00451$) between the absolute values of CD4+ T Lymphocytes and TReg were observed. In Group 2, a median age of 39.5 years and statistical dependence ($p=0.00234$) were observed between absolute values of low TReg lymphocytes in patients with high detectable viral load. In Group 3, the median age was 40.5 years and there was statistical dependence ($p = 0.00188$) between the absolute values of CD4+ T Lymphocytes and TReg Lymphocytes. CONCLUSION. Our data suggest that antiretroviral therapy increases the amount of total leukocytes and CD4+ T lymphocytes, presumably due to the increase in TReg lymphocytes.

Keywords: Regulatory T cells (Treg), human immunodeficiency virus, HIV, antiretroviral therapy.

Introduction

Acquired Immunodeficiency Syndrome is the final stage of human immunodeficiency virus (HIV) infection, marking the complete compromise of the individual's immune system, with an increase in the count of viral copies and heightened susceptibility to opportunistic infections[1,2,3,4,5]. Within this context, it is believed that much of the critical evolution of the HIV carrier's condition could be attributed to the response regulated by regulatory T lymphocytes (TReg). These cells sometimes contribute to maintaining homeostasis and individual physiological conditions, and at other times amplify disease pathogenesis, resulting in a decline in CD4+ T lymphocyte-mediated cellular immune response and viremia control [3,5,6,7,8]. However, the precise role of these cells in the clinical outcome of the infection still remains somewhat clear [9,10,11].

However, it is understood that the TReg lymphocytes represent a minority subset of CD4+ T lymphocytes that are capable of expressing CD4+ and CD25+ antigens (IL-2 receptor α chain), as well as the transcription factor Foxp3 (Forkhead Box P3), specialized in suppressing inappropriate immune responses (overreactive) and maintaining tolerance homeostasis during the course of several diseases[5,8,9,10,11,12,13,14].

In this context, a theory widely accepted among authors is that the elevation in the absolute concentration of TReg lymphocytes during the course of HIV infection leads to a reduction in viral load and a subsequent increase in the concentration of CD4+ lymphocytes[5,101,15,16] However, according to other authors, the increase in the absolute concentration of TReg lymphocytes appears to prevent an exaggerated immune response, which can promote virus replication and suppression of the immune response mediated by HIV-specific CD8+ T lymphocytes[8,11,12,17]. Chen et al[5] suggest that the count of TReg lymphocytes in individuals with HIV can serve as a potential marker for monitoring disease progression.

Therefore, the objective of this study was to assess the absolute concentration of TReg lymphocytes (CD4+CD25+FOXP3+) during the course of HIV infection in patients being diagnosed, both without specific treatment and those already receiving treatment and clinical follow-up.

Materials and methods

Casuistry

Cross-sectional and analytical study carried out with individuals aged 18 years and older who were undergoing diagnostic investigation, either without specific treatment or already receiving treatment and clinical follow-up for HIV infection, and who voluntarily visited a private laboratory in the city of Belém - Pará between July 2018 and August 2019 to receive quantification of CD4+ and T CD8+ lymphocytes, measurement of HIV viral load and blood count.

The individuals were categorized into three groups based on to their characteristics, as outlined: Group 1 (control), n=10, selected at random, with individuals who only received a routine blood count; Group 2, n=20, with individuals who received a Real Time Reverse Transcriptase Polymerase Chain Reaction test (RT-PCR) for HIV1/HIV2 and a complete blood count on the same day, but were not receiving treatment; and Group 3, n=20, with individuals who received a detectable RT-PCR test for HIV1/HIV2 and a complete blood count on the same day, and were already undergoing treatment (regardless of duration).

Ethical aspects

As this study involves no direct interaction between researchers and research subjects, as well as no personal data beyond gender and age, the researchers committed to preserving the confidentiality of acquired data by signing a Data Use, Confidentiality, and Storage Responsibility Agreement with the institution that provided the data. This procedure is aligned with the guidelines of Resolution No. 466/2012 of the National Health Council. To ensure anonymity, individuals were identified only by the numerical system from the laboratory that supplied the data.

Inclusion and Exclusion Criteria

A total of 107 patients were initially analyzed. However, 57 cases were excluded as they did not meet minimum criteria, such as not receiving a RT-PCR test and a blood count simultaneously on the study day or lacking a patient history of antiretroviral treatment. The gender of the subjects included in this research was not considered a criterion for data analysis. Individuals of all genders and age groups were included in the study.

Biological Samples

All samples analyzed originated from venous blood and were previously collected using the conventional technique. The collection was performed in tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant, with volumes of 4mL. The absolute leukocyte count (thousand/mm3) and the absolute (mil/mm3) and relative (%) lymphocyte count were determined using an electronic blood cell counter (BC-6800, Mindray →), following the manufacturer's instructions. T CD4+ and T CD8+ lymphocytes were quantified by flow cytometry, and viral load data were obtained. These data were obtained via remote access to the SOFTLAB laboratory management software, version 1.9, for the specified period.

These samples were separated and processed at the end of the day to determine the concentration of regulatory T lymphocytes (TReg)

CD4+CD25+FOXP3+ using flow cytometry.

Absolute Determination of TReg Lymphocytes

The process used 100µL of whole blood from each individual's sample, placed in individual tubes suitable for flow cytometry. To this, 5µL of surface monoclonal antibodies labeled with fluorochromes were added: anti-CD25+ FITC (fluorescein isothiocyanate), anti-CD3+ PE (phycoerythrin) and anti-CD4+ PercP (peridine-chlorophyll-protein complex). After that, the tubes were incubated under dim light and at room temperature for 10 minutes. Then, using the BD IntraSure → membrane permeabilization kit, 100µL of reagent A were added to the tubes and the samples were incubated for 15 minutes at room temperature in the dark. Afterward, 2mL of buffer solution (PBS) was added, and the samples were centrifuged for 3 minutes at 3000 rpm, followed by resuspension also in PBS. In the next step, 5µL of intracellular monoclonal anti-FoxP3 antibody labeled with the fluorochrome APC (allophycocyanin) was added, along with 100µL of reagent B, and the mixture was incubated again for 15 minutes at room temperature in the dark. After this period, 2mL of PBS was added, and the samples were centrifuged again for 3 minutes at 3000 rpm, followed by a new resuspension of the cells in PBS. Finally, all samples were acquired on a FACSCalibur® flow cytometer, and the data analysis was performed using the CellQuest Pro software (BD Biosciences) after recording 10,000 events.

Statistical analysis

Descriptive non-parametric methods were employed, including Fisher's Exact Test and a linear regression test using Pearson's Correlation Test in the GraphPad Prism 5 software (GraphPad, 2007). The statistical significance level was set at $p \leq 0.05$.

Results

A total of 50 samples from individuals of all genders and age groups were analyzed. The samples were categorized into three groups. Group 1 (control), n=10, consisted of samples of 5 men and 5 women, who were randomly selected and received routine tests. After selection, the samples underwent serological tests using enzyme-immunoassay (ELISA) to diagnose anti-HIV1/HIV2 antibodies, and the results were negative. Group 2, n=20, consisted of samples of 5 women and 15 men who received RT-PCR tests for HIV1/HIV2 and complete blood count on the same day, but were off treatment. Group 3, n=20, also consisted of samples from 5 women and 15 men who received RT-PCR tests for HIV1/HIV2 and complete blood count on the same day, but were already undergoing treatment (regardless of duration).

In group 1, the median age of individuals was 56.5 years, total leukocyte count was 7,690/mm3, the absolute count of CD4+ T Lymphocytes was 2,950/mm3 and the absolute count of TReg Lymphocytes was 323/mm3 (Table 1). As for the statistical analysis, a high statistical dependence ($p = 0.00451$) was observed solely between the absolute values of CD4+ T Lymphocytes and TReg Lymphocytes.

Table 1. Quantitative analysis of age, total leukocytes, CD4+ T lymphocytes and TReg lymphocytes of individuals from Group 1, randomly selected, who underwent routine blood count and negative serology for HIV1/HIV2 in a private laboratory in Belém - Pará , from July 2018 to August 2019.

	Age	LT (mm³)	CD4/CD3 (mm³)	CD4/FoxP3 (mm³)
Min - Max	17 - 96	4.280 – 8.890	1.818 – 4.468	88 - 783
Median	56,5	7.690	2.950	323
X ± DP	53,7 ± 24,8	7.089 ± 1.732,7	2.838 ± 853,1	360 ± 211,8

Legend: Min – Minimum; Max – Maximum; X – mean value; SD – Standard Deviation; - LT – Total Leukocytes; CD4/CD3 – Absolute Values of CD4 T Lymphocytes; CD4/FoxP3 – Absolute Values of TReg Lymphocytes.

In Group 2, the median age was 39.5 years, total leukocyte count was 6,220/mm3, the absolute count of CD4+ T Lymphocytes was 1,524/mm3, the absolute count of CD8+ T Lymphocytes was 804/mm3 and the absolute count of TReg Lymphocytes was 21/mm3 (Table 2). As for the statistical analysis, no statistical dependence was observed between the absolute values of total leukocytes, T CD4 + or T CD8 + lymphocytes in relation to the absolute count of TReg lymphocytes.

Subsequently, Fisher's exact test was employed to analyze the correlation between detectable or undetectable viral load and the increase or decrease in the absolute values of TReg lymphocytes for Group 2 (untreated). This analysis revealed a statistical difference (p=0.00234) between individuals with high detectable viral load and low absolute values of TReg lymphocytes.

Table 2. Quantitative analysis of age, total leukocytes, CD4+ T lymphocytes, CD8+ T lymphocytes, CD4/CD8 T Lymphocyte Ratio, TReg Lymphocytes of individuals in Group 2 who had detectable RT-PCR for HIV1/HIV2 and blood count on the same day, but they were out of treatment, from a private laboratory in Belém - Pará, from July 2018 to August 2019.

	Age	LT (mm³)	CD4/CD3 (mm³)	CD8/CD3 (mm³)	Ratio CD4/CD8	FoxP3/CD4 (mm³)
Min - Max	21 - 74	4.990 – 15.560	718 - 4.390	177 – 1.811	0,22 – 1,78	4 - 512
Median	39,5	6.220	1.524	804	0,61	21
X ± DP	43,4 ± 14,6	6.634 ± 1.616,1	1.589 ± 854,1	731 ± 366,6	0,688 ± 0,433	47,2 ± 131

Legend: Min – Minimum; Max – Maximum; X – mean value; SD – Standard Deviation; - LT – Total Leukocytes; CD4/CD3 – Absolute Values of CD4 T Lymphocytes; CD8/CD3 – Absolute Values of CD8 T Lymphocytes; CD4/CD8 – Absolute Values of the ratio of CD4 T Lymphocytes and CD8 T Lymphocytes; CD4/FoxP3 – Absolute Values of TReg Lymphocytes.

In Group 3, the median age was 40.5 years, total leukocyte count was 5,710/mm3, the absolute count of T CD4+ Lymphocytes was 1,435/mm3, the absolute count of CD8+ T Lymphocytes was 615/mm3 and the absolute count of TReg Lymphocytes was 205/mm3 (Table 3). As for the statistical analysis, a moderate statistical dependence (p = 0.01351) was observed between the absolute counts of

total leukocytes and of TReg lymphocytes, and a high statistical dependence (p=0.00188) was observed between the absolute counts of lymphocytes T CD4 + and TReg Lymphocytes.

Table 3. Quantitative analysis of age, total leukocytes, CD4+ T lymphocytes, CD8+ T lymphocytes, CD4/CD8 T Lymphocyte Ratio, TReg Lymphocytes of individuals in Group 3 who had detectable RT-PCR for HIV1/HIV2, complete blood count on the same day and already on antiretroviral treatment, from a private laboratory in Belém - Pará, from July 2018 to August 2019. HIV+ patients treated with antiretrovirals in reassessment.

	Age	LT (mm³)	CD4/CD3 (mm³)	CD8/CD3 (mm³)	Ratio CD4/CD8	FoxP3/CD4 (mm³)
Min - Max	22 - 76	4.310 – 9.050	174 - 2.344	239 – 1.103	0,27 – 1,85	20 - 887
Median	40,5	5.710	1.435	615	0,69	205
X ± DP	44,6 ± 14,7	5.889 ± 1.188,5	1.364 ± 491.4	576,8 ± 240,2	0,75 ± 0,375	246 ± 234,4

Legend: Min – Minimum; Max – Maximum; X – mean value; SD – Standard Deviation; - LT – Total Leukocytes; CD4/CD3 – Absolute Values of CD4 T Lymphocytes; CD8/CD3 – Absolute Values of CD8 T Lymphocytes; CD4/CD8 – Absolute Values of the ratio of CD4 T Lymphocytes and CD8 T Lymphocytes; CD4/FoxP3 – Absolute Values of TReg Lymphocytes.

Subsequently, Fisher's exact test was employed to investigate the correlation between detectable or non-detectable viral load and the increase or decrease in the absolute values of TReg lymphocytes for Group 3 (treated). This analysis revealed no statistically significant difference (p=0.88889) between these factors, suggesting that, among individuals undergoing treatment, the presence of not of a detectable viral load is not associated with the absolute count of TReg lymphocytes.

Discussion

This study found a direct relationship of dependence between the amount of CD4 + T lymphocytes and Reg T lymphocytes in the control group (Group 1) and the group of HIV patients already receiving specific treatment (Group 3). This finding suggests that the increase in total leukocytes and CD4+ T lymphocytes in HIV patients is contingent on antiretroviral treatment which, in turn, increases the number of T Reg lymphocytes.

This can be indirectly inferred when analyzing the results obtained from HIV patients without specific treatment (Group 2), which show that individuals with a high detectable viral load have low absolute counts of TReg lymphocytes, suggesting that the delay in initiating antiretroviral treatment may negatively impact the functionality of TReg lymphocytes in these patients.

Studies by Borrow[1], Chen et at[5] and Kervevan & Chakrabarti[6] mention that HIV develops mechanisms that appear to surpass the immune system's activity, thereby facilitating its proliferation. Therefore, as the infection advances, the depletion of T CD4+, T CD8+, naive and T Reg lymphocytes in the lymphoid tissues can be observed.

However, the immune function of T Reg lymphocytes in HIV patients is still a controversial topic and the underlying mechanisms remain poorly understood, even though, in some cases, these T Reg lymphocytes seem to play an important role in controlling immunopathology during the chronic phase of the disease[5, 6,13,19].

Wan et al[13] and Veiga-Parga et al[17], for instance, associate the frequency of T Reg lymphocytes with viral load and disease progression in HIV-infected individuals. According to these authors, the absolute number of T Reg lymphocytes in the circulating blood of HIV-infected individuals decreases in both the acute and chronic phases as the disease advances in severe cases, when compared to the findings in healthy individuals (controls).

Matavele Chissumba et al[20], Chen et al[5] and Moreno-Fernandez, Presicce & Chougnet[22], on the other hand, propose that chronic HIV infection may be associated with a relative increase in T Reg lymphocytes, either due to the "relative resistance" of these cells to the virus or due to their reduced susceptibility to apoptosis[8,23].

For other authors[6,12,13,19], however, these T Reg lymphocytes in acute HIV infection appear to prevent an exacerbated immune response and directly regulate the virus replication in CD4 + T cells. Conversely, in chronic HIV infection, these cells seem to favor virus proliferation and suppress the HIV-specific CD8 + T cell response[24].

Schulze et al[25], in turn, emphasize the importance of quantifying the total number of T Reg lymphocytes in HIV patients using absolute values, as, in their studies, the absolute count of these cells increased significantly with the initiation of antiviral therapy in all HIV-infected individuals when compared to controls. This observation is supported by Roider et al[11], who strongly suggest that the absolute count of T Reg lymphocytes can be an important indicator of the progression of HIV infection. Our findings further support these data, as we observed that individuals who had already initiated antiretroviral treatment had higher absolute counts of T Reg lymphocytes compared to patients who were not yet treated.

Conclusion

Our data support the observation made by other authors regarding the fact that the absolute increase in T Reg lymphocyte counts in patients receiving antiretroviral therapy enhances the count of total leukocytes and CD4+ T lymphocytes in people with HIV. However, there is still a clear need for further studies that can deepen the comprehension and ascertain the real importance of the absolute quantification of T Reg lymphocytes in the course of HIV infection as a method of clinical follow-up.

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