

## CLINICAL SCIENCE

# Thymopoiesis and regulatory T cells in healthy children and adolescents

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**OBJECTIVES:** The purpose of this study was to investigate the association between T cell receptor excision circle levels in peripheral blood mononuclear cells and regulatory T cells that co-express CD25 and Foxp3 in healthy children and adolescents of different ages.

**MATERIALS AND METHODS:** The quantification of signal-joint T-cell receptor excision circle levels in the genomic DNA of peripheral blood mononuclear cells was performed using real-time quantitative PCR. The analysis of CD4, CD8, CD25, and Foxp3 expression was performed using flow cytometry.

**RESULTS:** Ninety-five healthy controls (46 females and 49 males) ranging in age from 1 to 18 years were analyzed. The mean T-cell receptor excision circle count in all individuals was  $89.095 \pm 36.790$  T-cell receptor excision circles per microgram of DNA. There was an inverse correlation between T-cell receptor excision circles counts and age ( $r = -0.846$ ;  $p < 0.001$ ) as well as between the proportion of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells and age ( $r = -0.467$ ;  $p = 0.04$ ). In addition, we observed a positive correlation between the amount of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells and the amount of T-cell receptor excision circles per microgram of DNA in individuals of all ages ( $r = -0.529$ ;  $p = 0.02$ ).

**CONCLUSIONS:** In this study, we observed a decrease in the thymic function with age based on the fact that the level of T-cell receptor excision circles in the peripheral blood positively correlated with the proportion of regulatory T cells in healthy children and adolescents. These findings indicate that although T-cell receptor excision circles and regulatory T cells levels decrease with age, homeostasis of the immune system and relative regulatory T cells population levels are maintained in the peripheral blood.

**KEYWORDS:** T lymphocytes; Thymus; Foxp3; T-cell Receptor.

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## INTRODUCTION

The thymus provides a unique microenvironment for T-cell receptor (TCR) rearrangement and plays a crucial role in immune system homeostasis and in maintaining tolerance to self antigens. The thymus is responsible for the positive selection of major histocompatibility complex-restricted T cells, the negative selection of self-reactive immune cells and the differentiation of several distinct T-cell subsets with immunoregulatory properties, such as regulatory T (Treg) cells, natural killer (NK) cells and intraepithelial lymphocytes. The thymus is fully developed at birth and undergoes a life-long process of involution until advanced ages (1-3).

Several methods have been used to evaluate thymic function in the peripheral blood. The quantification of TCR excision circles (TREC) in peripheral mononuclear blood cells has been widely used as an indirect marker of recent thymus emigrant (RTE) cells (4,5). TRECs are episomal DNA circles generated during intra-thymus T-cell maturation. These products are relatively stable and do not undergo replication during mitosis; therefore, TRECs become diluted with each round of cellular replication in the peripheral T cells (6). Few studies have evaluated thymic function in healthy children. However, these studies have shown that TRECs are present at relatively high levels in the peripheral T cells during the first years of life, reflecting an elevated rate of thymopoiesis during childhood, which decreases dramatically with age (7-9).

The determination of TREC levels in peripheral blood mononuclear cells has been widely conducted to estimate the number of RTEs in several situations, such as during antiretroviral therapy in children and adults with HIV infection and after allogeneic hematopoietic stem cell

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transplantation (10-12). In addition, the quantification of TRECs is considered a useful tool to estimate the rate of thymopoiesis in newborns with primary immunodeficiencies and DiGeorge syndrome as well as in autoimmune diseases such as juvenile idiopathic arthritis (13-14).

Regulatory T cells (Treg) play an important role in the maintenance of the self-tolerance and homeostasis of the immune system. Treg cells are defined by the expression of the cell surface markers CD4 and CD25 and by the intracellular expression of the transcription factor Foxp3. Foxp3 is strongly associated with the regulatory function of Treg cells (15-17). The contribution of Treg cells to immune system homeostasis is best illustrated by the spontaneous development of autoimmune diseases in mice that are deficient in the Foxp3 gene. In addition, children born with a dysfunctional Foxp3 gene develop IPEX/XLAAD (immunodysregulation, polyendocrinopathy, and enteropathy X-linked syndrome/X-linked autoimmunity-allergic dysregulation) (18).

Studies evaluating the rate of thymopoiesis relative to the proportion of Treg cells in healthy children are important for better understanding the mechanisms involved in the regulation and development of certain juvenile autoimmune diseases. However, these types of studies remain scarce. In the present study, we evaluated the association between the number of TREC copies and the proportion of Treg cells in the peripheral blood of healthy children and adolescents of different ages.

## MATERIAL AND METHODS

### Subjects

This study was conducted at the Children's Hospital, University of São Paulo (USP) and at the UNIFESP Medical School Hospital. Ninety-five clinically healthy children and adolescents between the ages of 1 and 18 years living in the urban center of São Paulo, Brazil, were selected for the study. The patients were recruited during routine pediatrician outpatient visits or during preoperative evaluation for minor surgical procedures, such as postectomy and inguinal hernia, at the UNIFESP Medical School Hospital. All healthy control patients underwent thorough clinical evaluations and were screened for a normal blood cell count. Exclusion criteria for the healthy controls included prior thymectomy, the presence of signs or symptoms of primary immunodeficiency disease based on the "10 Warning Signs of Primary Immunodeficiency" (Jeffrey Modell Foundation), current prescription medication use, and the presence of active bacterial or viral infections. Atopic children and those with premature birth were also excluded. This study was approved by the local Ethics Committee of HCFMUSP (CAPPesq). Informed written consent was obtained from the parents of all children included in the study.

### Methods

Peripheral venous blood samples (5-10 mL) were collected using EDTA Vacutainer blood collection tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Paque-Plus (GE Healthcare, Uppsala, Sweden) density gradient centrifugation according to the manufacturer's suggested protocol. PBMC samples were frozen in aliquots for subsequent DNA extraction (-20°C) and flow cytometry analysis (-80°C). Genomic DNA was precipitated from all blood samples using a salting-out method (19). The

DNA concentration and purity were determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). Signal-joint TREC (sjTREC) concentrations were analyzed by real-time quantitative PCR (Rotor Gene 3000, Corbett Research Pty Ltd., Sydney, Australia) using SYBR Green reagent (DyNAmo SYBR Green qPCR Kit F400L, Finnzymes, Finland). The PCR primer sequences used for qRT-PCR were as follows: 5'-CCCTTCAACCATGCTGACA-3' (sense) and 5'-AGGTG-CCTATGCATC ACCGT-3' (antisense). In each reaction, the DNA sample was also tested in duplicate for  $\beta$ -actin transcript levels as an amplification control. The PCR primers for the  $\beta$ -actin transcript were as follows: 5'-TCACCCACACTGTGCCCATCTACGA-3' (sense) and 5'-CAGCGGAACCGCTCATTGCCAATGG-3' (antisense). The PCR protocol included an initial denaturation step for 10 minutes at 95°C, which was followed by 45 cycles of 15 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°C.

A standard curve was included in every PCR reaction for the absolute quantification of the number of sjTRECs per  $\mu$ g of DNA in each sample. The TREC standard curve was established using seven 10-fold dilutions that ranged from  $10^2$  to  $10^8$  TREC copies/ $\mu$ L of plasmids containing an sjTREC fragment.

Characterization of the T-cell subsets was performed by flow cytometry using frozen PBMCs. Samples were thawed rapidly in a 37°C water bath. Once thawed, the cells were counted, and  $5 \times 10^6$  cells were stained with ECD-conjugated anti-CD3 (Beckman Coulter), APC.Cy7-conjugated anti-CD4 (BD Pharmingen), and PE.Cy7-conjugated anti-CD8 (BD Pharmingen) antibodies. To determine the percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells, PBMCs were stained with a human Treg cell staining kit (eBioscience) according to the manufacturer's protocols. The kit contained PE-conjugated anti-Foxp3 PCH101, FITC-conjugated anti-CD4, and APC-conjugated anti-CD25. The samples were analyzed using a FACS Canto II flow cytometer (BD Biosciences). The data were analyzed using FlowJo software (TreeStar).

**Statistical analysis.** To investigate the normal distribution of TREC copies, the Kolmogorov-Smirnov test was used. The Mann-Whitney test was used to compare quantitative variables, and Spearman's correlation coefficient was used to correlate quantitative variables. All analyses were performed using the SPSS statistical software version 15.0. A p-value less than 0.05 was considered statistically significant in all analyses.

## RESULTS

Ninety-five healthy children and adolescents were analyzed. The mean age of the patients was 9 years (range: 1-18 years). The mean TREC count in PBMCs in all individuals was  $8.9 \times 10^4 \pm 3.6 \times 10^4$  TRECs/ $\mu$ g of DNA. The Kolmogorov-Smirnov test revealed that the TREC content of our samples did not display a normal distribution. There were no statistically significant differences in the average TRECs/ $\mu$ g of DNA between female and male patients ( $8.2 \pm 3.3 \times 10^4$  TRECs/ $\mu$ g of DNA versus  $9.5 \pm 3.9 \times 10^4$  TRECs/ $\mu$ g of DNA, respectively;  $p=0.085$ ). Healthy patients between 1 and 9 years of age had significantly higher TREC levels than healthy patients between 10 and 18 years of age ( $11.2 \times 10^4$  TREC/ $\mu$ g of DNA versus  $6.5 \times 10^4$  TREC/ $\mu$ g of DNA, respectively;  $p<0.001$ ) (Table 1). As shown in Figure 1, there

**Table 1** - Number of TREC copies/ $\mu\text{g}$  DNA and relative expression of  $\text{CD3}^+$ ,  $\text{CD4}^+$ ,  $\text{CD8}^+$  T cells and regulatory T cells ( $\text{CD4}^+\text{CD25}^+\text{Foxp3}^+$ ) in healthy children and adolescents among different age groups (median  $\pm$  standard deviation).

	Total (n = 95)	1 - 9 years (n = 47)	10 - 18 years (n = 48)	p-value (1-9 versus 10-18 years)
TREC/ $\mu\text{g}$ DNA	89.095 $\pm$ 36.79	112.706 $\pm$ 38.71	65.975 $\pm$ 11.71	0.001
$\text{CD3}^+$ (n = 63)	52 $\pm$ 11%	51.3 $\pm$ 10%	52.4 $\pm$ 11%	0.41
$\text{CD4}^+$ (n = 63)	63.4 $\pm$ 10.2%	62.6 $\pm$ 11%	64.4 $\pm$ 8.6%	0.55
$\text{CD8}^+$ (n = 63)	16.4 $\pm$ 10.3%	15.26 $\pm$ 8.9%	17.5 $\pm$ 8.9%	0.31
$\text{CD4}^+\text{CD25}^+\text{Foxp3}^+$ (n = 19)	3.7 $\pm$ 2.3%	4.7 $\pm$ 1.5%	3.2 $\pm$ 2.5%	0.17

was a strong inverse correlation between age and TREC counts in PBMCs in all individuals ( $r = -0.846, p < 0.001$ ).

The proportion of  $\text{CD3}^+$ ,  $\text{CD4}^+$ , and  $\text{CD8}^+$  cells in 63 children and adolescents ranged from 45 to 62%, 60 to 65%, and 14 to 26%, respectively (Table 1). There was no significant correlation between the proportion of  $\text{CD3}^+$ ,  $\text{CD4}^+$ , and  $\text{CD8}^+$  cells and patient age ( $p = 0.424, r = 0.103; p = 0.908, r = 0.015; p = 0.372, r = 0.114$ , respectively).

The mean percentage of  $\text{CD4}^+\text{CD25}^+\text{Foxp3}^+$  T cells was  $3.7 \pm 2.3\%$ . There was an inverse correlation between  $\text{CD4}^+\text{CD25}^+\text{Foxp3}^+$  T cells and patient age ( $r = -0.467; p = 0.04$ ). In addition, we found a positive correlation between the expression of Treg cells and the number of TRECs/ $\mu\text{g}$  of DNA ( $r = 0.529; p = 0.02$ ) (Figure 2).

**DISCUSSION**

The thymus plays a crucial role in immune system homeostasis and in Treg cell production. There has been an increased interest in the function of the thymus and in the relative contribution of the different T-cell subsets in normal and pathological contexts. In the present study, TREC levels were significantly increased in the peripheral blood of children from 1 to 3 years of age, and, in agreement with previous studies, there was a progressive decrease in TREC levels as the patient age increased (7,8). We also observed a significant decrease in the proportion of Treg cells ( $\text{CD4}^+\text{CD25}^+\text{Foxp3}^+$ ) as the patient age increased. Finally, we observed a positive correlation between TREC levels and the proportion of Treg cells in the peripheral blood.

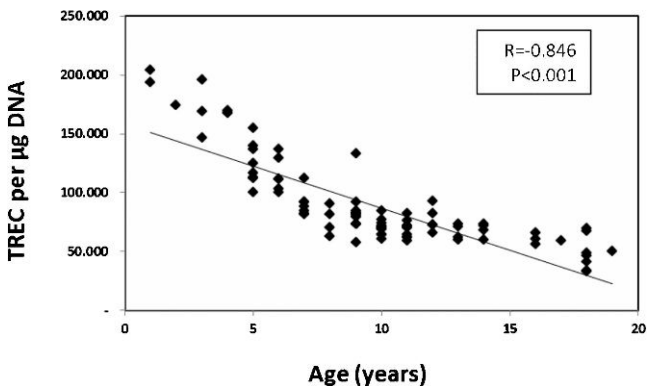
Previous studies have demonstrated high TREC levels in cord blood and in the peripheral blood of young children (12,9). The decline in thymic output begins at birth and increases at a more rapid rate as age increases (7). Nevertheless, TRECs are measurable even in older individuals, indicating that the thymus remains active in older individuals (7,8,11). Interestingly, we found variability in the number of TREC copies in children and adolescents of the same age. This variability has been previously observed and has been attributed to genetic factors and inherent differences in the proportion of thymic tissue subtypes in healthy patients of the same age (4,11). In addition, epigenetic factors have been proposed to affect thymus function and TREC levels. In agreement with previous studies, the TREC levels observed in our study were similar among male and female children and adolescents (7).

The amount of TREC copies in the peripheral blood has been studied in several autoimmune diseases and primary and secondary immunodeficiencies, particularly in the pediatric population. Children with juvenile idiopathic arthritis have low TREC levels in the peripheral blood (14,20). In HIV-infected children and adults, TREC levels have been used to evaluate the reconstitution of immune function following antiretroviral therapy (21).

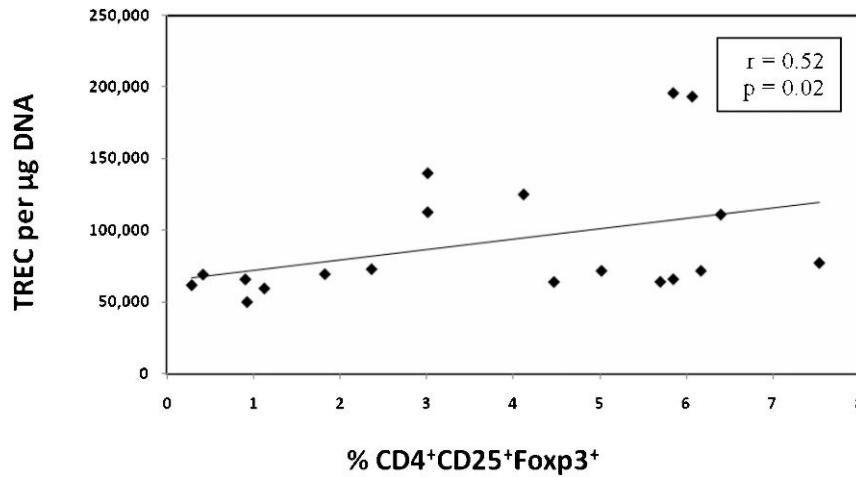
In primary immunodeficiency diseases, different types of thymus involvement are responsible for several abnormalities in immune function. Athymic children with DiGeorge syndrome have undetectable or very low TREC levels. After transplantation of thymus tissue in children with DiGeorge syndrome and following stem cell or bone marrow transplantation in SCID, a gradual increase in TREC levels in the peripheral blood is typically observed, which is indicative of the gradual reconstitution of the immune system (9,22,23). Recently, TREC quantification has been proposed as a screening test for the early diagnosis of SCID in newborns, highlighting the importance of TREC quantification in certain immunodeficiencies (24,25).

Nonetheless, several factors other than thymic output can affect TREC levels in PBMCs, such as peripheral T-cell proliferation, peripheral T-cell death, and T-cell redistribution (26,27). Particularly in pathological situations, we believe that proliferation and apoptosis markers should be considered in parallel with TREC levels to assess immunodeficiencies.

In this study, the characterization of  $\text{CD3}^+$ ,  $\text{CD4}^+$ , and  $\text{CD8}^+$  T cells was used to provide a more comprehensive picture of the relative levels of the different T-cell subtypes in the peripheral blood. In addition, the characterization of the T-cell subsets was the initial step in evaluating Treg levels in the peripheral blood. We found similar proportions of  $\text{CD3}^+$ ,  $\text{CD4}^+$ , and  $\text{CD8}^+$  T cells to those found in previous studies (28). Furthermore, we did not find any correlation



**Figure 1** - Distribution of TREC levels per  $\mu\text{g}$  of DNA among healthy children and adolescents from 1 to 18 years of age. TREC levels per  $\mu\text{g}$  of DNA showed a strong inverse correlation with age ( $r = \text{Spearman's correlation coefficient}$ ).



**Figure 2** - Distribution of TREC levels per µg of DNA and CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cell levels. TREC levels per µg of DNA showed a positive correlation with the level of regulatory T cells (r = Spearman's correlation coefficient).

between the levels of CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T-cell subtypes and patient age.

Treg cells represent a unique lineage of immunoregulatory cells in humans and animals and play a central role in the maintenance of immunological self-tolerance. Treg cells can be divided into two groups: thymus-derived natural Treg (nTreg) cells and peripheral-induced adaptive Treg (aTreg) cells. Despite distinct developmental roles, both nTreg and aTreg cells express Foxp3, which contributes to their suppressive function. In our study, we considered Treg cells as the CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> population because the transcription factor Foxp3 is considered highly specific for these cells (29). Recent data suggest that nTreg cells may be identified by the high expression of another transcription factor called Helios (30). Helios expression could be used in future studies to differentiate nTreg cells from aTreg cells, which do not express Helios. In our study, we found that an average of 3.7% of the total T-cell population consists of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells, which is in agreement with previous reports (31,32).

We also found a moderate inverse correlation between the expression of Treg cells and age in the children and adolescents studied. Several studies have evaluated the correlation between age and Treg cell levels in healthy children; however, the results of these studies are conflicting. Some studies have reported higher proportions of Treg cells in newborns, indicating that Treg cells may be stimulated when children are exposed to foreign antigens (33,34). Gridebacke et al. (33) and Fichizawa et al. (32) found increased expression of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells in the peripheral blood during the first few days of life, followed by stabilization in the level of Treg cells during the first years of life. Furthermore, Teran et al. (34) evaluated a group of children from Latin America and found a significant decrease in the relative amount of Treg cells as age increased. Furthermore, similar to our study, Teran et al. also found variability in the Treg levels in individuals of the same age, indicating that factors such as environment may affect the development of the regulatory arm of the immune system.

Our study revealed for the first time a positive correlation between thymopoiesis, as indicated by the number of TREC copies, and the expression of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells in the peripheral blood. T-cell homeostasis is heavily regulated.

Treg cells play a key role in maintaining self-tolerance and autoimmune regulation. As previously mentioned, the absence of Treg cells due to genetic manipulation or depletion results in the development of many autoimmune diseases. Furthermore, a decrease in the rate of thymopoiesis is associated with the gradual loss of T-cell repertoire diversity, which also increases the risk of autoimmunity. Based on our collective results, we postulate that Treg cells in healthy children can be proportionally exported from the thymus and positively correlate with an RTE marker.

The number of Treg cells found in an individual is influenced by other factors besides thymus output. The maintenance of Treg cells in the periphery is a dynamic process that is influenced by genetic factors, the proliferation rate of Treg cells, and the movement of Treg cells to inflammatory sites. The mechanisms involved in this complex inter-relationship are beginning to be revealed, and new studies will clarify the importance of each factor on Treg cell homeostasis.

In the present study, we established normal values of TREC levels in the peripheral mononuclear T-cell population in healthy children and adolescents of different ages. In agreement with previous studies, we showed a significant decrease in thymic function with age. We also observed an inverse correlation between CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells and age. Furthermore, our study revealed a positive correlation between Treg cells and TREC levels in the periphery. Collectively, our results indicate that although TREC and Treg cell levels decrease during the aging process, immune system homeostasis and normal relative Treg cell populations are maintained in the periphery. Future studies will involve applying our findings to children who are affected by autoimmune diseases and immunodeficiencies to better understand the mechanisms involved in the development of these diseases. These studies will aid in the development of novel therapeutic interventions for these conditions.

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## AUTHOR CONTRIBUTIONS

Arisмени MA performed all molecular and immunological experiments. Kallás EG was responsible for the operation of the Flow Cytometry Laboratory LIM-60 (Laboratório de Investigação Médica LIM 60). Santos BA helped to conduct the flow cytometry experiments. Carneiro-Sampaio MM was responsible for the operation of the Molecular Biology Laboratory LIM-36 (Laboratório de Investigação Médica LIM 36). Kayser C was the principal investigator.

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