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Original Article

Evaluation of thymopoiesis in healthy Turkish children aged 0-6 years

Akif Kavgacı[1], Deniz Bayrakoğlu[2], Sevgi Köstel Bal[2], Şule Haskoloğlu[2], Nisa Eda Çullas İlarslan[1], Seda Topçu[3], Emel Okulu[4], Candan İslamoğlu[2], Meltem Arıkan[2], Figen Doğu[2], Kamile Aydan İkincioğulları[2]

[1]Department of Pediatrics, Ankara University Faculty of Medicine, Ankara; [2]Department of Pediatric Immunology and Allergy, Ankara University Faculty of Medicine, Ankara; [3]Department of Social Pediatrics, Ankara University Faculty of Medicine, Ankara; [4]Department of Neonatology, Ankara University,Faculty of Medicine, Ankara, Türkiye.

ABSTRACT

Background. Early diagnosis and effective treatment serve as life-saving procedures for primary immunodeficiencies (PIDs) which are very common and a major public health problem in Turkey. Severe combined immunodeficiency (SCID) is constitutively a T-cell defect in which naïve T-cell development is defective due to the mutations in genes responsible for the T cell differentiation and insufficient thymopoiesis. So, assessment of thymopoiesis is very important in the diagnosis of SCID and several combined immune deficiencies (CIDs).

Methods. The purpose of this study is to examine thymopoiesis in healthy children via measurement of recent thymic emigrants (RTE); T lymphocytes that express CD4, CD45RA and CD31 to establish the RTE reference values in Turkish children. RTE were measured in the peripheral blood (PB) of 120 healthy infants and children between 0-6 years including cord blood samples, by flow cytometry.

Results. The absolute count of RTE cells and their relative ratios were found to be higher during the first year of life, being highest at the 6th month and tending to decrease significantly by age following birth (p=0.001). In the cord blood group, both values were lower than those in the 6-month-old group. The absolute lymphocyte count (ALC) varying by age, was found to reduce to 1850/mm^3 in 4-years and after.

Conclusions. Here we evaluated normal thymopoiesis and established the normal reference levels of RTE cells in the peripheral blood of healthy children aged between 0-6 years. We believe that the collected data will contribute to early diagnosis and monitoring of immune reconstitution; serving as an additional fast and reliable marker for many PID patients especially for SCID including many other CIDs, especially in nations where newborn screening (NBS) via T cell receptor excision circles (TREC) has not yet become available.

Key words: recent thymic emigrants (RTE), thymopoiesis, flow cytometry.

Primary immune deficiencies (PIDs) are a heterogeneous group of diseases that emerge as a result of mutations in the genes that code the components of the immune system. According to the classification of the International Union of Immunological Societies (IUIS) that was revised in 2020, immunodeficiencies are divided into 10 groups.[1] Due to the higher rates of parental consanguinity in Turkey, combined immunodeficiencies (CID) inherited as an autosomal recessive pattern are seen more frequently.[2,3] Among CID diseases, severe combined immunodeficiencies (SCID) are a pediatric emergency that result in mortality in cases where it is not diagnosed early and treated appropriately. SCID is mainly a

✉ Akif Kavgacı
akifkavgaci@gmail.com

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developmental disorder of T-cells. About 20 genetic defects that lead to this disease have been defined.[4] Screening of newborn patients during their asymptomatic period will provide opportunities for early diagnosis and treatment. Early diagnosis and treatment may be lifesaving in SCID and other CIDs.

Absent or low autologous naïve T-cell count is the hallmark of SCID and in most CID due to abnormal and insufficient thymopoiesis. T cell receptor excition circles (TREC) measurement is the most reliable and appropriate method in the diagnosis of SCID and CID. TREC is an indicator of thymopoiesis, and it has been used in the United States of America since 2008 for screening of SCID and early diagnosis of it from heel blood samples collected from newborns.[5]

Today, thymopoiesis, namely the function of the thymus gland, can be measured by the evaluation of recent thymic emigrants (RTE). RTE can be assessed by two different methods. The first is the enumeration of TREC copy numbers via molecular assay which involve the measurement of DNA residues with circular structures that remain from the recombination of V(D)J genes during the molecular development of T-cell receptors. The second is the measurement of naïve T cell levels expressing CD4, CD45RA and CD31 together on their surfaces by flow cytometry. These cells have high TREC contents.

Since peripheral blood (PB) lymphocyte subtype analysis is a very important and informative tool for the diagnostic immune work-up in most PIDs mainly CID, RTE enumeration should also be included in the basic diagnostic panel of flow cytometric analysis of PIDs.

This study aims to examine thymopoiesis in a cross-sectional manner in healthy children from birth to age 6 years and to obtain RTE reference levels and counts by enumerating CD4, CD45RA, CD31 expressing T cells in PB in healthy children between 0-6 years. We believe that the collected data will contribute to early diagnosis and monitoring of immune reconstitution serving as an additional fast and reliable marker for many PID patients especially for CID including SCID, particularly in nations where NBS via TREC has not become available yet.

Material and Methods

Characteristics of Healthy Subjects

A total of 120 healthy children aged between 0-6 years who had normal developmental features and were free from any sign or symptom of acute infections; 20 in each of 6 subgroups (cord blood, 6 months, 1 year, 2 years, 4 years and 6 years) were included to the study during the period of February 2017 to December 2017.

Those who had a past personal history or family history of PID or other chronic diseases receiving immunosuppressive agents were excluded from the study.

Cord blood samples were collected during normal vaginal delivery of term at gestation newborns who were born to pregnant women without any known chronic disease or infection at the Gynecology and Obstetrics Department. Those with a history of Cesarean section or perinatal asphyxia (5th-minute APGAR score of <7), those with clinical (hypo-hyperthermia, apnea-respiratory disorder, lethargy, color change in the skin) and laboratory (leukopenia <5000/mm^3 or leukocytosis >25000/mm^3) left-shifted peripheral blood smear (immature-to-total neutrophil ratio of >20%) finding indicating infection or those with infection proven by a positive culture, those with Hb values of<12.5 gr/dl and anemia and/or requirement of blood exchange, those with ABO and Rh-Rh incompatibility and infants with congenital anomalies were excluded from the study.

This study was approved by the Ethics Committee of Ankara University on 23.01.2017. Written informed consent was obtained from the parents of all infants and children.

Peripheral Blood (PB) Sampling

2 ml of peripheral blood or cord blood was collected to Ethylenediaminetetraacetic acid (EDTA) containing tubes in all children and given to the laboratory immediately. Whole blood count (leukocytes and absolute lymphocyte counts (ALC) and RTE levels were measured simultaneously in all samples.

Flow Cytometric Analysis of RTE

The RTE measurements were carried out via flow cytometer (NAVIOS, Beckman Coulter, USA) by 4-color staining using the fresh PB samples and whole blood lysis method at the Immunology Research Laboratory of the Department of Pediatric Immunology-Allergy at Ankara University Medical School. In the CD4+ cell population within the CD45/SS gate, CD45RA+CD31+ cell levels were measured. Flow cytometric data were analyzed by Kaluza 1.3 software (Beckman Coulter, USA).

Statistical Analysis

The data were analyzed at the Department of Biostatistics at Ankara University Medical School by using the IBM SPSS Statistics 15.0 (IBM Corp., Armonk, New York, USA) software. The descriptive statistics included frequencies (n), percentages (%), mean ± standard deviation, medians, 5th percentile and 95th percentile values. Pearson's chi-square and Fisher's exact tests were used to analyze the categorical variables. The normal distribution of the numerical variables was analyzed by the Shapiro Wilk normality test and Q-Q plots. Two groups were compared by independent-samples t-test for the normally distributed variables and by Mann Whitney U test for the non-normally distributed variables. $p<0.05$ was accepted to be statistically significant.

Results

The age and sex characteristics of the age-based 6 subgroups are shown in Fig. 1. The median and 5-95% intervals of white blood cell, absolute lymphocyte counts and CD4+ T cell percentages of all children are given in Table I.

The absolute lymphocyte count (ALC) was also found to vary based on age. In the 6-month-old and 1-year-old age groups, ALC was 3132/mm³ and 3056/mm³ at the 5% lower limit

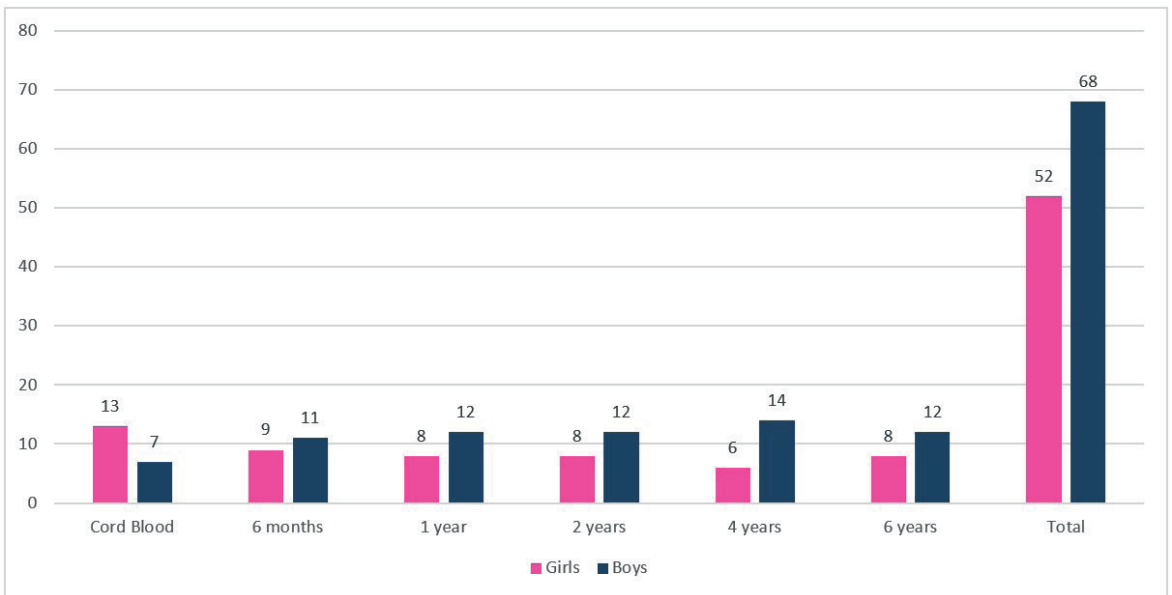

Fig. 1. The age and sex characteristics of 6 subgroups.

Table I. WBC, ALC and CD4+ T cell percentages in peripheral blood based on age.

		CB	6 months	1 year	2 years	4 years	6 years	p
WBC/mm³	Median	12935	8535	9410	8260	8215	7940	p=0.001
			p=0.001	ns	ns	ns	ns	
	5%	5967	5901	5770	6521	4652	4640	
	95%	27545	17071	13356	11555	11922	16508	
ALC/mm³	Median	3930	5260	5385	4865	3395	3105	p=0.001
			p=0.012	ns	ns	p=0.002	ns	
	5%	2484	3132	3056	2870	1850	1881	
	95%	8949	12092	9652	7747	5784	7244	
CD4+ T cells (%)	Median	50.68	51.55	45.60	39.80	39.45	39.83	p=0.001
			ns	ns	ns	ns	ns	
	5%	27.22	33.63	32.43	28.26	28.38	30.59	
	95%	63.07	62.35	58.69	51.23	52.04	51.08	

* p>0,05: ALC: absolute lymphocyte count, CB: cord blood, ns: not significant, WBC: white blood cells.

respectively, decreasing with the increase of age, and reducing to 1850/mm³ in the 4-year-old group and after.

The median and 5-95% intervals of relative rates of CD4+CD45RA+CD31+ cells (RTE) and absolute counts of RTE cells in all children are given in Table II.

The absolute counts of RTE and their relative rates (%) significantly (p=0.001) decreased by age; found to be highest during the first year of life, especially the 6th month (median 77% and 2243/mm³), the decrease gradually reaching median 58% and 732/mm³ (69 - 73% and 1654-406/mm³) at 6 years. The absolute count and percentage value (relative rates) of the RTE cell levels in the cord blood group were found to be lower than those in the 6-month-old group, respectively (p=0.004, p=0.001) (Fig. 2 and Fig. 3)

Table II. Absolute RTE counts and RTE percentages in peripheral blood based on age.

		CB	6 months	1 year	2 years	4 years	6 years	p
RTE (%) CD4+ CD45RA+ CD31+	Median	68.36	76.85	73.00	68.90	63.97	58.05	p=0.001
			p=0.001	ns	ns	ns	ns	
	5%	59.66	63.59	57.15	57.19	55.46	53.73	
	95%	81.09	81.79	82.37	79.36	74.47	69.89	
Absolute RTE/mm³	Median	1319	2043	1829	1377	852	732	p=0.001
			p=0.012	ns	ns	p=0.002	ns	
	5%	896	929	969	646	544	406	
	95%	2299	4981	3832	2284	1516	1654	

* p>0,05: CB: cord blood, ns: not significant, RTE: recent thymic emirgants.
Severe Combined Immunodeficiency (SCID) [Internet]. Available from: https://esid.org/Working-Parties/Clinical/Resources/Severe-Combined-Immunodeficiency-SCID.

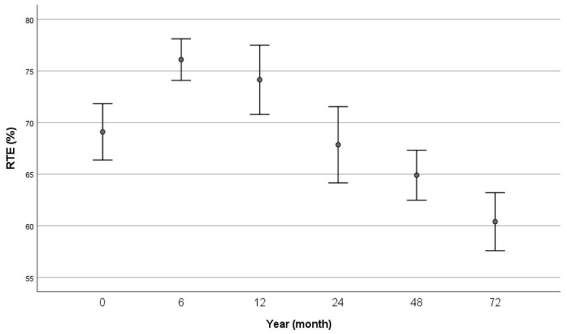

Fig. 2. Age-dependent change in recent thymic emigrant (CD4+CD45RA+CD31+) cell levels.

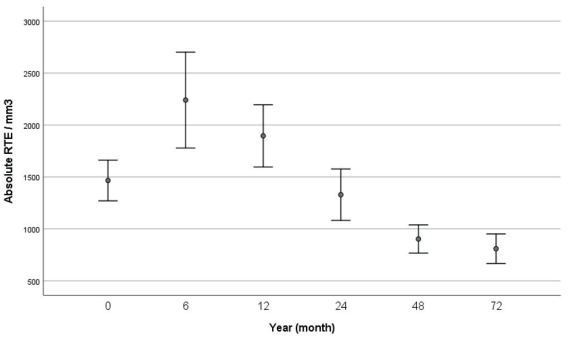

Fig. 3. Age-dependent change in absolute counts of recent thymic emigrant (CD4+CD45RA+CD31+) cells.

Discussion

TREC measurement by RT-PCR or RTE (CD4CD45RA+CD31+) measures by flow cytometry are used to assess thymopoiesis in severe combination immunodeficiency and several other combined immunodeficiencies using DNA extracted from heel blood. In our country, SCID is not yet included in the newborn screening program. Compared to the molecular enumeration of TREC by RT-PCR, the identification of RTE by flow cytometry is much faster and more valuable for the diagnostic workup of PID suspected patients. There is no information on reference values of RTE in healthy Turkish children. The aim of this study was to identify normal RTE levels in children aged 0-6 years.

In the literature, there are limited data measured in healthy infants and children, that would allow for commenting on whether the percent and absolute counts that are obtained as a result of measuring thymopoiesis immunophenotypically are normal or low. NBS of SCID with RTE measurement, was not found to be cost-effective due to the high cost and requirement of more blood samples, flow cytometry devices, and experienced personnel.[6] However, it is well known by clinical immunologists that at the stage of decision, reference values obtained from age-matched healthy children are needed for an accurate diagnostic approach. Thus, we believe that the data obtained in our study will be helpful to us and other clinical immunology staff in this respect and promote early diagnosis in SCID and CID patients.

Another result we obtained in this study was that the ALC values varied by age. In the 6-month-old and 1-year-old age groups, ALC was found to be 3132/mm³ and 3056/mm³ at the 5% lower limit, while it expectedly decreased in older age groups, and it was reduced to 1850/mm³ in the 4-year-old group and after as the 5% lower limit.

It was also shown in this study yet again that the lymphopenia limit in the first year of life is 3000/mm³ and an absolute lymphocyte count of less than 3000 per mm³ in a newborn can be used as the cutoff for consideration of a T-cell disorder.[7] Absolute lymphocyte count is age-dependent and as opposed to the neonatal period, from the age of 1 on, values under 1500/mm³ are considered lymphopenia. Six of 11 screening programs in the United States defined significant T-cell lymphopenia as a T-cell count less than 1500/μL.[8] However, the data in our study showed that ALC decreases gradually by age, and the 5% lower limit of ALC at the age of 4 years and later is 1850/mm³. In the light of these data, it was shown that the lower limit of lymphopenia was higher than 1500/mm³, which is the generally accepted lower limit, and ALC should be carefully evaluated in patients with suspicion of PID.

Schatorjé et al.[9] demonstrated that absolute T lymphocyte numbers increase 1.4-fold during the first months of life, and after 9–15 months,

they decrease threefold to adult values; this is mainly caused by the expansion of recent thymic emigrants and naive cells. Helper and cytotoxic T lymphocytes show the same pattern. In our study, PB RTE absolute counts and percentage values were significantly lower in the cord blood group in comparison to the 6-month-old group. Based on the reviews, it was also reported in the literature that the CD45RA+ naïve T-cell relative and absolute rates peak in the 6th postpartum month, and these levels in the cord blood are lower in comparison to those in 6-month-olds.[10] Not unexpectedly, our data show that RTE follow the pattern of naive helper T lymphocytes after the neonatal period. This difference in the absolute counts and relative rates of RTE cells may have been caused by the unique composition of cord blood. Nevertheless, the lymphocyte levels in cord blood are lower in comparison to newborns and breastfeeding infants.[11]

As we emphasized earlier, RTE cells contain a high level of TREC and there are several studies in the literature. Junge et al.[12] demonstrated that TREC content in CD31+CD45RA+CD4+T cells was 18 times higher than in CD31–CD45RA+CD4+lymphocytes. Garcia-Prat et al.[13] measured TREC levels with the PCR method and reported that these levels tended to decrease with age. In the study where the correlation between CD4+ naive T-cell levels and RTE and TREC was also examined, it was reported that the correlation between the CD4+ naïve T-cells and RTE (r^2 = 0.4160) was more significant than that with TREC (r^2 = 0.3013). In this study, a correlation between TREC and RTE values was detected.

With increasing age, the absolute counts of RTE cells (p=0.001) and their relative ratios (p=0.001) decreased. The main reason for this change is the structural change in the thymus tissue and involution by age. In the cross-sectional study by Schatorjé et al.[9] which examined the stages of major T-cell differentiation in the childhood period, the study emphasized that RTE increased by 1.6 times in the first 5 months of life, and then, it reached adult values by decreasing down to an eightfold. It was reported that the CD4+ T-cells of a newborn included more RTE in comparison to those in adults, and RTEs followed the distribution of naïve T lymphocytes after the newborn period.

It is known that due to the higher rates of parental consanguinity in Turkey, CID inherited as an autosomal recessive pattern are seen more frequently. The study, which was conducted in Turkey in 2019 and evaluated the usability of CD31 in the evaluation of thymic production, included 66 children with suspected immunodeficiency based on clinical and laboratory findings. The most common diagnoses among the patients were humoral immunodeficiencies (34.8%), combined immunodeficiencies (34.8%) and patients with thymus aplasia/hypoplasia or thymectomy due to cardiac surgery (7.6%). The percentages of RTE did not differ statistically between these groups (p=0.231), but the absolute numbers of RTEs were significantly lower in the CIDs group (p=0.007). At least one cardiac pathology was observed in 36.9% (n: 24) of the patients in this study. Eight patients had corrective cardiac surgery before the study. RTE cell relative ratios and absolute counts were lower in the operated group, respectively (p=0.011, p=0.032). This data shows that similar to the decrease in RTE relative ratios and absolute counts due to the development of thymic involution with age, damage to thymic tissue also causes a decrease in RTE relative ratios and absolute counts.[14]

Allogenic hematopoietic stem cell transplantation (HSCT) is mainly a curative treatment in some certain PID. The objective of HSCT in PID is to create a new and normally operating immune system by forming stable and permanent engraftment and immune reconstitution (IR). For exactly this purpose, inspection and monitoring of IR in patients after the transplantation is one of the factors that play a significant role in determining the prognosis. Likewise, longitudinal monitoring of thymopoiesis by measurement of RTE levels

after transplantation contributes greatly to the assessment of the IR after HSCT and the determination of the prognosis. In this case, the normal values obtained in this study from healthy infants and children aged 0-6 years may allow objective assessment of immune constitution after HSCT and thymopoiesis.

Similarly, the RTE cell levels in the peripheral blood of 22 SCID patients who received allogenic HSCT between the years 2008-2014 and were monitored for two years at the Department of Pediatric Immunology-Allergy at the Faculty of Medicine at Ankara University were retrospectively analyzed by examining CD4+CD45RA+CD31+ cells. It was discovered that after HSCT, RTE cell levels increased noticeably in all patients, regardless of donor type or immunophenotype, from the first to the sixth month after transplantation, and remained high and stable from the 6th month to the 2nd year. The study emphasized that measuring RTE levels in peripheral blood by measuring CD4+CD45RA+CD31+ cells in peripheral blood is a valuable indicator in assessing and monitoring immune reconstitution after HSCT.[15]

Consequently, this study in which we assessed the RTE (CD4+CD45RA+CD31+) cell levels of 120 healthy infants and children is in the position of being the first study that has been conducted in Turkey on this topic. The normal values of the RTE cells in the peripheral blood of healthy Turkish children as well as in the cord blood of 0-6 year old age groups were determined. In Turkey, where primary immunodeficiencies constitute a significant health problem, we believe that assessment of the RTE cell levels and counts of patients with pre-diagnosis of PID by comparison to the normal values of these age groups will significantly contribute to the early diagnoses and treatments of patients. Hence, the determination of CD4+CD45RA+CD31+ cells should be included in the peripheral blood lymphocyte sub-group panels at all centers that perform the diagnosis and treatment of PID patients.

Ethical approval

The study was approved by the Ethics Committee of Ankara University (Number: 02-61-17, date: 23.01.2017).

Author contribution

The authors confirm contribution to the paper as follows: Study conception and design: AK, Aİ; data collection: NECI, ST, EO, CI; analysis and interpretation of results: DB, MA, AK, SKB, SH, Aİ; draft manuscript preparation: AK, SH, FD, Aİ. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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