Original Article

Extended Immunophenotyping Reference Values in a Healthy Pediatric Population

Marina Garcia-Prat,[1,2] Daniel Álvarez-Sierra,[1] Aina Aguiló-Cucurull,[1] Sandra Salgado-Perandrés,[1] Sara Briongos-Sebastian,[1] Clara Franco-Jarava,[1] Andrea Martin-Nalda,[2] Roger Colobran,[1,3] Isabel Montserrat,[4] Manuel Hernández-González,[1,3] Ricardo Pujol-Borrell,[1,3] Pere Soler-Palacin,[2] and Mónica Martínez-Gallo[1,3]*

[1]Immunology Division, Hospital Universitari Vall d'Hebron (HUVH), Vall d'Hebron Research Institute (VHIR), Barcelona, Catalonia, Spain

[2]Pediatric Infectious Diseases and Immunodeficiencies Unit, Hospital Universitari Vall d'Hebron (HUVH), Vall d'Hebron Research Institute (VHIR), Universitat Autònoma de Barcelona (UAB), Barcelona, Catalonia, Spain

[3]Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona (UAB), Barcelona, Catalonia, Spain

[4]Hematology Division, Hospital Universitari Vall d'Hebron (HUVH), Vall d'Hebron Research Institute (VHIR), Barcelona, Catalonia, Spain

Background: For the accurate diagnosis of immunodeficiencies is crucial to compare patients' immunology laboratory values with age-sex matched controls, yet there is a paucity of normal values for most populations.

Objectives: To define appropriate reference values of extended lymphocyte subpopulations and T-cell receptor excision circle (TRECs) levels in healthy pediatric donors between 1 month and 18 years of age.

Methods: Extended immunophenotyping values were obtained by analysis of multiparameter flow cytometry panels for the following subpopulations: CD4+ and CD8+ Naive, Effector, Effector Memory and Central Memory, T helper subpopulations and their degrees of activation, T Regulatory cells, Recent Thymic Emigrants (RTE), B Lymphocyte subpopulations (Transitional, Naive, Preswitch-Memory, Switch-Memory, Plasmablasts, CD21low, and Exhausted), and subpopulations for Monocytes, NK cells and Dendritic Cells.

Results: Median values and the 10th and 90th percentiles were obtained for 32 lymphocyte and monocyte subpopulations, and for TRECs levels in each age group of children. Naive CD4+ and CD8+ T-cell populations tended to decrease with age, with significant difference between the groups, in parallel with the reduction in thymic function assessed by TRECs counts and the recent thymic emigrant population. Relative numbers of Th cell populations tended to increase with age. The percentage of class-switched B cell populations showed a significant increase between the youngest group and the others.

Conclusion: This study provides essential data for interpreting extended immunophenotyping profiles in the pediatric and young adult populations, which could be of value for the diagnosis of PIDs and immune-mediated diseases, particularly those associated with subtle immunological abnormalities.

Key terms: Immunophenotyping; pediatric; reference values; flow cytometry; primary immunodeficiencies; peripheral blood lymphocyte subpopulations

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Abbreviations: DC, dendritic cells; NK, natural killer; PID, primary immunodeficiency; RTE, recent thymic emigrant; RVs, reference values;TCM, central memory T cells TEM effector memory T cells; TEMRA, terminal effector T cells CD45RA+; Th, T helper cell;TRECs, Tell receptor excision circles; TReg, T regulatory cell

***Correspondence to:** Dr. Mónica Martínez-Gallo. Servei d'Immunologia, Edifici Laboratoris Clínics, planta baixa. Hospital Universitari Vall d'Hebron. Passeig Vall d'Hebron 119-129, 08035 Barcelona, Spain. Email: mmartinez@vhebron.net

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INTRODUCTION

Immunophenotyping of blood lymphomononuclear cell populations is usually performed by analyzing the expression of cell surface markers (clusters of differentiation) by flow cytometry. This provides valuable information on the distribution and state of activation of these cells among the large number of defined subpopulations. The circulating cellular compartment of the human immune system shows the most marked shifts during the first years of life, but is also dynamic during adult life, showing a tendency to a reduction in function and diversity with age (1). When an immune system disorder is suspected, immunophenotyping of the patient's peripheral blood mononuclear cells (PBMCs) is one of the initial steps in the diagnostic work-up, but reliable reference values (RVs) for lymphocyte subpopulations are often lacking, particularly for those recently introduced in the clinical diagnosis, and this hinders the interpretation of the results.

Primary immunodeficiencies (PIDs) are a heterogeneous group of genetic disorders quantitatively or qualitatively affecting the immune system that includes more than 300 entities, with an incidence of approximately 1:2,000 live newborns. The Expert Committee of the International Union of Immunological Societies has classified PIDs into nine groups based on the clinical manifestations and laboratory immunological abnormalities (2). Prompt identification of PID patients reduces complications and is associated with a more favorable prognosis (3). In addition to a complete medical history, physical examination, and general laboratory tests, the initial evaluation protocol when PID is suspected includes analysis of serum immunoglobulin levels and extended immunophenotyping in peripheral blood. Immunophenotype abnormalities can range from a complete absence of a specific cell population to more subtle variations in the differentiated states of specific subpopulations (4,5). It is known that levels of the main lymphocyte subpopulations vary during the first years of life; for example, CD3+ T cells increase immediately after birth and decrease gradually with increasing age (6). Hence, RVs should be available for each of the well-established peripheral blood lymphocyte, dendritic cell, and monocyte subsets in healthy children in different age ranges, particularly those that are good indicators of their maturation and activation states. PIDs are predominantly pediatric diseases. Nonetheless, data from the European Society for Immunodeficiencies (ESID) registry (www.ESID.org) have shown that almost half of all patients are diagnosed as they approach adulthood. Therefore, RVs stratified by age are also needed for adolescents.

Although RVs for the most common lymphocyte subpopulations have been reported in pediatric patients (7,8), these data are not valid as general RVs for the diagnosis of hematological and immunological disorders for several reasons: (i) limited number of blood samples from children aged less than 2 years, (ii) 10th and 90th percentiles are unavailable for all cell populations, (iii) data are missing for many of the recently defined memory T cell populations, and (iv) incomplete data on reagents, staining protocols, instrument settings, and acquisition and analysis procedures (9–11). The lack of consensus in the stratification by age and the criteria for including healthy donors are factors precluding use of the available RVs. In the light of these shortcomings, it is important to define the RVs for immune cell subpopulations following state of the art protocols in a large enough population of well-defined healthy individuals stratified by age in such a way that the main differences resulting from age-related biological changes are highlighted.

The Human Immune Phenotyping Consortium (HIPC) program developed by the Federation of Clinical Immunology Societies (FOCIS) promotes standardization of flow cytometry immunophenotyping in clinical studies. The HIPC panels and standard operating procedures were designed to define most of the well-established and clinically significant immune cell subsets in peripheral blood mononuclear cells (PBMCs) (T cells, regulatory T cells, T helper 1/2/17 cells, B cells, natural killer cells, dendritic cells, and monocytes). This strategy is applicable for use in multicenter clinical trials, allows detection of relative and absolute numbers of nearly all blood leucocyte subsets, and has proven to be robust and instrument-independent (12).

Here, we report the RVs for an extended immunophenotyping panel in 6 age groups of healthy children using the HIPC program protocol. As CD31+CD45RA+RO- lymphocytes (recent thymic emigrant cells [RTE]) contain large numbers of T cell receptor circle (TREC)-bearing T cells, we also investigated the age-related correlations between RTE, TRECs, and CD4+ naive T cells, data of particular interest for groups performing hematopoietic stem cell transplantation. The data presented should be of value for laboratories performing extended phenotype studies following the FOCIS protocols in patients with immune-mediated diseases.

SUBJECTS AND METHODS

Subjects and Blood Collection

In total, 159 samples from healthy pediatric donors were included [81 males (51%) and 78 females (49%); aged 1 month to 18 years]. There is no ethnic segregation. Samples were obtained in Hospital Universitari Vall d'Hebron, (Barcelona, Spain), with approval of the hospital ethics committee (PR_AG_134-2011). All parents/legal guardians of the children included were appropriately informed and consent was obtained. Children with a history of immune system disorders or syndromes affecting the immune system, receiving immunosuppressive therapy, or blood-derivative transfusion in the previous year were excluded from the study. All participants were registered anonymously and stratified by age and sex.

Blood was collected in vacutainer tubes containing ethylene-diamine-tetra-acetic acid (EDTA) as anticoagulant (BD-Plymouth, PL6 7BP, UK) and processed within 4 h after collection.

Multicolor Staining and Analysis

Extended lymphocyte subpopulations were assessed with five different panels designed according to the HIPC protocol (12). Two additional panels were added to analyze the basic lymphocyte populations and RTE. We adapted the protocol designed for the BD LSRFortessa instrument to the BD FACSCanto II (Becton Dickinson). Compensation controls were used in each panel to avoid overlapping of the different fluorochromes. Details of the panels and gating strategies are provided in Supporting Information Figure S1. The samples were stained according to the numbered panels as follows: Panel 1—General immune phenotype of T, B, and natural killer (NK) lymphocyte subpopulations, gating by CD45 versus SSC. Panel 2—Gating strategy for differentiated CD4+ and CD8+ T-cell subsets, based on CD45RA and CCR7 expression defining: CD45RA+/CCR7+ (naïve), CD45RA−/CCR7+ (central memory [TCM]), CD45RA−/CCR7− (effector memory [TEM]), and CD45RA+/CCR7− (terminal effector memory [TEMRA]). CD4+ T-helper (Th) populations (Th1, Th2, Th17, Th1–17), based on CCR6 and CXCR3 expression, were analyzed by gating on CD45RA− TCM and TEM cells. Panel 3—T-regulatory (Treg) cell populations: CD3+CD4+CD25+, CD127−, CCR4+, and CD45RO+. Panel 4—B-cell populations (naïve, pre-switched, switched memory, and exhausted) depending on expression of IgD and CD27. The differing pattern of CD24+ and CD38+ expression identified transitional cells and plasmablasts. CD27 and CD21 enabled study of the CD21low population. Panel 5—Dendritic cells (DC), NK cells, and monocyte populations were analyzed in the CD3−CD19− gate. NK subpopulations (NK dim and NK bright) were studied using CD56 and CD16 expression. The markers CD16 and CD14 were used to identify classical monocytes (CD14+CD16−) and non-classical monocytes (CD16+CD14−). DCs were studied selecting the population negative for the following markers: CD3, CD14, CD16, CD19, CD20, and CD56. High expression of HLA-DR and CD11c and CD123 was used to identify plasmacytoid DCs (HLA-DR+CD123+) and myeloid DCs (HLA-DR+CD11c+). Panel 6—RTEs were studied using CD3, CD4, CD27, CD31, CD45RA, and CD62L expression.

Peripheral whole blood (50 μL) was incubated with a mix of specific conjugated monoclonal antibodies (mAb) from each panel and gently mixed for 20 min at room temperature (RT) in the dark. The composition of mAb mixes, concentrations, clones, and brands are specified in Table 1. Samples were treated with 1 mL of lysing solution (BD FACS lysing solution), vortexed, and incubated for 15 min at RT in the dark. Samples were then washed with phosphate-buffered saline (PBS), stored at RT in the dark, and analyzed within 1 h; the samples were acquired with a FACS Canto II (Becton Dickinson) flow cytometer, equipped with three lasers: a 405-nm violet laser, a 488-nm blue laser, and a 647-nm red laser. At least 100,000 events were acquired from each sample. Flow cytometry data were analyzed with BD FACSDiva v8.0.1 software (BD Biosciences). Internal quality assurance procedures included BD cytometer setup and tracking beads, according to the manufacturer's instructions.

Absolute values were calculated from the absolute number of leucocytes and lymphocytes provided by the hematological analyzer (XN-2000; Sysmex, Japan).

Quantification of T-Cell Receptor Excision Circles

TRECs quantification was carried out by qPCR using TaqMan probes. The input sample was 100 ng of gDNA from whole peripheral blood. Results are expressed as TRECs/100 ng of DNA. A reference gene segment (ribonuclease P) was amplified in parallel to ensure that there was no bias in DNA quantification or quality. qPCR was performed using the TaqMan 7900 Fast Real-time PCR System (Applied Biosystems). Reactions were run in triplicate in a 96-well plate. The sequences of the primers and probes and description of the reaction conditions are available on request.

Statistics

Subjects were stratified into six age groups, each with equal representation of males and females. Age intervals were from 1 month to 2 years, 3–4, 5–6, 7–8, 9–13, and 14–18 years. The number of individuals in each age group varied from 15 to 40 (n = 159). A descriptive analysis was done for each of the qualitative variables (sex) and quantitative variables (cell population) (data not shown).

For each lymphocyte population, we defined a normal range based on the median, and the 10th and 90th percentiles of cell frequencies, according to the variable "age range" in SPSS. Each variable was analyzed in both absolute number and percentage.

Data analysis used the Graph Pad Prism software, version 5.04. Distribution of the data was assessed by the D'Agostino and Pearson omnibus normality tests.

Table 1
Monoclonal Antibodies Used for Extended Immunophenotype

Panel	Monoclonal Antibody	Fluorochrome	Clone	Brand, City	Volume (μL)
(1) General Lymphocytes populations	CD3	FITC	SK7	Becton Dickinson (BD), New Jersey	20
	CD8	PE	SK1		
	CD45	PerCP	2D1 (HLe-1)		
	CD4	APC	SK3		
	CD3	FITC	SK7	Becton Dickinson (BD), New Jersey	20
	CD16	PE	B73.1		
	CD56	PE	NCAM 16.2		
	CD45	PerCP	2D1 (HLe-1)		
	CD19	APC	SJ25C1		
(2) T-cell populations	CD3	Pacific Blue	UCHT1	Tonbo Biosciences, San Diego	5
	CD4	PerCP·Cy5.5	RPA-T4	Tonbo Biosciences, San Diego	5
	CD8	APC·H7	SK1	Immunostep, Salamanca	5
	CCR7	PE	3D12	BD, New Jersey	10
	CD45RA	FITC	L48	BD, New Jersey	5
	CD3	Pacific Blue	UCHT1	Tonbo Biosciences, San Diego	5
	CD4	PerCP·Cy5.5	RPA-T4	Tonbo Biosciences, San Diego	2.5
	CD8	APC·H7	SK1	Immunostep, Salamanca	2.5
	CXCR3	APC	G025H7	BioLegend, San Diego	2.5
	CCR6	PE·Cy7	G034E3	BioLegend, San Diego	5
	HLA-DR	FITC	L243°	BD, New Jersey	10
(3) T regulatory cell population	CD3	Pacific Blue	UCHT1	Tonbo Biosciences, San Diego	5
	CD4	PerCP·Cy5.5	RPA-T4	Tonbo Biosciences, San Diego	5
	CD25	PE	2A3	BD, New Jersey	20
	CD127	APC	A019D5	BioLegend, San Diego	5
	CCR4	PE.Cy7	1G1	BD, New Jersey	10
	CD45RO	APC-H7	UCHL1	BD, New Jersey	5
	HLA-DR	FITC	L243	BD, New Jersey	20
(4) B-cell populations	CD19	PerCP-Cy5.5	HIB19	Tonbo Biosciences, San Diego	2.5
	CD27	PE-Cy7	O323	Tonbo Biosciences, San Diego	2.5
	CD21	Pacific Blue	HI21a	Immunostep, Salamanca	2.5
	IgD	FITC	IA6–2	BD, New Jersey	5
	IgM	APC-Cy7	MHM-88	BioLegend, San Diego	5
	CD24	PE	ML5	BD, New Jersey	2.5
	CD38	APC	HIT2	BD, New Jersey	5
(5) DC/Monocytes/NK	CD3	APC-H7	33-2A3	Immunostep, Salamanca	5
	CD19	APC-H7	HIB19	BD, New Jersey	5
	CD14	Pacific Blue	47-3D6	Immunostep, Salamanca	5
	CD56	PE	N901(HLDA6)	Beckman Coulter, California	20
	CD16	APC	3G8	Immunostep, Salamanca	20
	HLA-DR	FITC	L243	BD, New Jersey	20
	CD123	PerCP-Cy5.5	7G3	Immunostep, Salamanca	5
	CD11c	PE-Cy7	B-LY6	Immunostep, Salamanca	5
(6) Recent Thymic Emigrants	CD3	APC	UCHT-1	Cytognos, Salamanca	5
	CD4	PerCP	L200	BD, New Jersey	5
	CD45RA	PE Cy7	HI100	Immunostep, Salamanca	5
	CD31	FITC	WM59	BD, New Jersey	5
	CD27	VA450	O323	Tonbo Biosciences, San Diego	5
	CD62L	PE	DREG-56	BD, New Jersey	5

Differences between the age groups were evaluated by the Kruskal–Wallis method with Dunn's multiple comparison test. A *P*-value of <0.05 was considered statistically significant. Correlations between TRECs, RTEs, CD4+ naive were analyzed by linear regression between pairs of parameters.

RESULTS

Reference Values for Lymphocyte Subpopulations in Healthy Children

The median and 10th and 90th percentiles (P10, P90) of absolute counts, and relative size of the main lymphocyte populations (T and B lymphocytes, NK cells) were obtained for the six age groups (Table 2). It is known that the total lymphocyte percentage in circulating leucocytes decreases with age. Hence, as was expected, total lymphocyte counts were highest in the first 2 years of life (median 5.42; P10 3.26 to P90 8.84) and gradually decreased over time to adult values (median 2.50, P10 1.37 to P90 3.47) with significant differences between the age groups ($P < 0.0001$). Regarding the specific lymphocyte populations, T cells showed a reduction in absolute count, but the percentages of CD4+ and CD8+ T cells remained stable. In the case of B cells, both the relative percentage and absolute count declined with age, with significant

Table 2
Absolute and Relative Size of the General Lymphocyte Populations

Subpopulation	Age groups																
	0–2 years (n = 24)			3–4 years (n = 20)			5–6 years (n = 15)			7–8 years (n = 26)			9–13 years (n = 40)			14–18 years (n = 34)			P
	Median	P10	P90	Median	P10	P90	Median	P10	P90	Median	P10	P90	Median	P10	P90	Median	P10	P90	
Lymphocytes (%)	**57**	*36*	*74*	**45**	*32*	*68*	**48**	*32*	*59*	**39**	*21*	*51*	**38**	*29*	*47*	**32**	*21*	*43*	**<0.0001**
Lymphocytes (×10^9/L)	**5.42**	*3.26*	*8.84*	**3.28**	*2.43*	*6.06*	**2.99**	*2.13*	*4.5*	**2.74**	*1.75*	*3.46*	**2.4**	*1.71*	*3.06*	**2.5**	*1.37*	*3.47*	**<0.0001**
CD3+ (%)	**65**	*52*	*77*	**69**	*58*	*80*	**69**	*57*	*77*	**71**	*61*	*84*	**70**	*60*	*79*	**71**	*62*	*81*	**0.1562**
CD3+ (×10^9/L)	**3.58**	*1.85*	*5.96*	**2.36**	*1.5*	*3.87*	**1.92**	*1.42*	*3.12*	**1.88**	*1.36*	*2.74*	**1.64**	*1.07*	*2.27*	**1.74**	*1.09*	*2.6*	**<0.0001**
CD4+ (%)	**40**	*30*	*58*	**41**	*30*	*50*	**42**	*24*	*46*	**39**	*26*	*53*	**41**	*29*	*48*	**42**	*31*	*53*	**0.8124**
CD4+ (×10^9/L)	**2.08**	*1.14*	*3.8*	**1.33**	*0.88*	*2.36*	**1.12**	*0.54*	*1.84*	**0.98**	*0.66*	*1.61*	**0.88**	*0.64*	*1.29*	**0.99**	*0.56*	*1.45*	**<0.0001**
CD8+ (%)	**20**	*12*	*27*	**21**	*16*	*31*	**23**	*16*	*33*	**25**	*19*	*35*	**23**	*18*	*33*	**26**	*19*	*30*	**0.0009**
CD8+ (×10^9/L)	**1.02**	*0.54*	*1.97*	**0.76**	*0.41*	*1.28*	**0.58**	*0.47*	*1.2*	**0.65**	*0.44*	*1.05*	**0.54**	*0.38*	*0.88*	**0.59**	*0.32*	*0.96*	**<0.0001**
CD19+ (%)	**22**	*15*	*28*	**17**	*9*	*31*	**17**	*12*	*25*	**14**	*8*	*23*	**14**	*8*	*21*	**12**	*6*	*21*	**<0.0001**
CD19+ (×10^9/L)	**1.14**	*0.64*	*1.96*	**0.59**	*0.31*	*1.13*	**0.5**	*0.26*	*0.97*	**0.32**	*0.2*	*0.68*	**0.33**	*0.17*	*0.63*	**0.32**	*0.14*	*0.56*	**<0.0001**
CD56 + CD16+ (%)	**11**	*3*	*24*	**10**	*5*	*23*	**12**	*6*	*21*	**14**	*6*	*21*	**13**	*8*	*22*	**16**	*6*	*23*	**0.112**
CD56 + CD16+ (×10^9/L)	**0.54**	*0.15*	*1.33*	**0.39**	*0.15*	*0.81*	**0.34**	*0.18*	*0.51*	**0.32**	*0.15*	*0.51*	**0.33**	*0.17*	*0.53*	**0.32**	*0.15*	*0.7*	**0.036**

differences between the groups ($P < 0.0001$). As to NK cells, their relative contribution to the total lymphocyte population remained stable with age, even though their absolute counts showed a tendency to decrease in parallel to that of CD3+ cells. There were no significant differences in the overall lymphocyte populations between sexes as seen by previous works (13) with the exception of CD4+ in relative numbers, being higher in females than in males in 9–13 years of age group ($P < 0.05$) (Data not shown).

Results for the lymphocyte populations analyzed following the adapted HIPC-protocol panels are shown in Table 3 (percentage and absolute cell count). Regarding the CD4+ and CD8+ T cell differentiation subsets (naive, TCM, TEM, and TEMRA), the percentages and absolute cell counts of the naive subpopulation tended to decrease with age, with significant differences between the groups. The TEM subpopulation percentage increased significantly with age, although there were no differences in terms of absolute numbers (Fig. 1). No significant differences were observed in the relative numbers of TCM and TEMRA subpopulations between groups. The CD4+ and CD8+ absolute counts showed the same tendency; TEMRA and TEM subpopulations remained stable, whereas TCM and naive subpopulations tended to decrease with age, with significant differences between the age groups.

The T-helper cell populations (Th1, Th2, Th17, Th1–17) were analyzed within the TEM plus TCM subpopulations. Relative numbers of Th1, Th17, and Th1–17 tended to increase, with significant differences between the age groups (Fig. 1). There were no differences in the Th2 subpopulation according to age. The activation status as assessed by HLA-DR expression in Th subpopulations was slightly higher in the youngest groups, and decreased with age. Other T cell subpopulations were analyzed: TReg displayed a slight tendency to increase with age, with significant differences between the youngest age group and the groups older than 7 years (7–8, 9–13, and 14–18 years). The percentage and absolute counts of RTE were, as expected, highest in the youngest age group, with significant differences compared with the oldest groups.

With regard to B cells, both the percentage and absolute number of naive CD19+ cells were highest in the youngest age group (1 months to 2 years) and gradually decreased thereafter, with a significant difference between the youngest group and the rest of groups (Fig. 2C). CD19+ transitional cells were analyzed by gating within CD19+ naïve cells. The percentage of this population decreased with age, and showed significant differences between the oldest group (14–18 years) and the other groups (Fig. 2B). The same occurred in the analysis of absolute numbers. Regarding the percentage of class switched B-cell populations (Fig. 2D), a significant increment was observed between the youngest group and the others although no significant differences were observed between the youngest group (0–2 years) and the 5–6 years old group. Of note, absolute

Table 3
Median and 10th–90th Percentiles of Extended Lymphocyte Populations Percentage

	Age groups																		
	0–2 years (n = 24)			3–4 years (n = 20)			5–6 years (n = 15)			7–8 years (n = 26)			9–13 years (n = 40)			14–18 years (n = 34)			
Subpopulation	Median	P10	P90	Median	P10	P90	Median	P10	P90	Median	P10	P90	Median	P10	P90	Median	P10	P90	P
CD4+ Naive %	**66**	*54*	*80*	**62**	*46*	*72*	**62**	*35*	*69*	**50**	*32*	*68*	**51**	*25*	*63*	**44**	*31*	*57*	**<0.0001**
CD4+ Naive (×10^9/L)	**1.43**	*0.6*	*3*	**0.81**	*0.5*	*1.6*	**0.63**	*0.2*	*1.2*	**0.48**	*0.2*	*1*	**0.47**	*0.1*	*0.7*	**0.48**	*0.3*	*0.7*	**<0.0001**
CD4+ Effector TEMRA (%)	**6**	*3*	*12*	**7**	*3*	*14*	**7**	*4*	*22*	**8**	*4*	*15*	**7**	*4*	*24*	**6**	*4*	*12*	**0.127**
CD4+ Effector TEMRA (×10^9/L)	**0.12**	*0.1*	*0.3*	**0.08**	*0*	*0.3*	**0.08**	*0*	*0.3*	**0.08**	*0*	*0.2*	**0.07**	*0*	*0.2*	**0.06**	*0*	*0.1*	**0.0129**
CD4+ Effector Memory %	**8**	*3*	*16*	**16**	*9*	*20*	**14**	*10*	*30*	**18**	*9*	*32*	**22**	*12*	*30*	**24**	*12*	*44*	**<0.0001**
CD4+ Effector Memory (×10^9/L)	**0.19**	*0.7*	*0.3*	**0.2**	*0.1*	*0.4*	**0.18**	*0.1*	*0.3*	**0.17**	*0.1*	*0.4*	**0.21**	*0.1*	*0.3*	**0.25**	*0.1*	*0.6*	**0.0354**
CD4+ Central Memory %	**15**	*10*	*26*	**16**	*11*	*20*	**16**	*9*	*25*	**18**	*9*	*24*	**18**	*11*	*25*	**21**	*10*	*27*	**0.0325**
CD4+ Central Memory (×10^9/L)	**0.31**	*0.2*	*0.7*	**0.22**	*0.1*	*0.4*	**0.17**	*0.1*	*0.3*	**0.17**	*0.1*	*0.3*	**0.15**	*0.1*	*0.3*	**0.2**	*0.1*	*0.3*	**<0.0001**
CD8+ Naive %	**57**	*34*	*73*	**50**	*29*	*72*	**44**	*23*	*68*	**49**	*30*	*61*	**41**	*22*	*58*	**43**	*18*	*61*	**0.0079**
CD8+ Naive (×10^9/L)	**0.53**	*0.2*	*1.1*	**0.37**	*0.2*	*0.6*	**0.29**	*0.1*	*0.6*	**0.26**	*0.2*	*0.6*	**0.22**	*0.1*	*0.4*	**0.24**	*0.1*	*0.4*	**<0.0001**
CD8+ Effector TEMRA %	**11**	*7*	*25*	**12**	*5*	*25*	**14**	*6*	*30*	**14**	*7*	*26*	**12**	*7*	*26*	**12**	*5*	*20*	**0.7268**
CD8+ Effector TEMRA (×10^9/L)	**0.11**	*0*	*0.3*	**0.09**	*0*	*0.3*	**0.07**	*0*	*0.3*	**0.09**	*0*	*0.2*	**0.07**	*0*	*0.2*	**0.07**	*0*	*0.2*	**0.1061**
CD8+ Effector Memory %	**22**	*9*	*47*	**30**	*14*	*49*	**32**	*14*	*59*	**31**	*20*	*45*	**39**	*24*	*58*	**39**	*25*	*58*	**0.0002**
CD8+ Effector Memory (×10^9/L)	**0.22**	*0.1*	*0.7*	**0.24**	*0.1*	*0.4*	**0.24**	*0.1*	*0.5*	**0.21**	*0.1*	*0.4*	**0.23**	*0.1*	*0.4*	**0.2**	*0.1*	*0.6*	**0.994**
CD8+ Central Memory %	**7**	*3*	*15*	**6**	*2*	*13*	**5**	*4*	*11*	**6**	*2*	*12*	**4**	*2*	*15*	**6**	*3*	*12*	**0.3016**
CD8+ Central Memory (×10^9/L)	**0.07**	*0.3*	*0.2*	**0.04**	*0.2*	*0.2*	**0.04**	*0*	*0.1*	**0.04**	*0*	*0.1*	**0.03**	*0*	*0.1*	**0.03**	*0*	*0.1*	**0.0003**
Th1%	**9**	*3*	*17*	**12**	*6*	*17*	**10**	*8*	*18*	**13**	*6*	*22*	**14**	*9*	*21*	**17**	*11*	*28*	**<0.0001**
Th1 (×10^9/L)	**0.17**	*0.1*	*0.3*	**0.14**	*0.1*	*0.3*	**0.14**	*0.1*	*0.2*	**0.14**	*0*	*0.2*	**0.12**	*0.1*	*0.2*	**0.16**	*0.1*	*0.2*	**0.0121**
Th2%	**9**	*5*	*14*	**9**	*6*	*15*	**9**	*7*	*19*	**11**	*5*	*14*	**10**	*8*	*14*	**11**	*7*	*16*	**0.2548**
Th2 (×10^9/L)	**0.15**	*0.1*	*0.4*	**0.11**	*0.1*	*0.2*	**0.1**	*0.1*	*0.2*	**0.1**	*0.1*	*0.2*	**0.1**	*0.1*	*0.1*	**0.11**	*0.1*	*0.2*	**0.0001**
Th1–17%	**3**	*1*	*6*	**4**	*2*	*7*	**5**	*3*	*10*	**6**	*3*	*10*	**8**	*5*	*14*	**11**	*7*	*19*	**<0.0001**
Th1–17 (×10^9/L)	**0.06**	*0*	*0.1*	**0.06**	*0*	*0.1*	**0.06**	*0*	*0.1*	**0.07**	*0*	*0.1*	**0.07**	*0*	*0.2*	**0.1**	*0.1*	*0.2*	**<0.0001**
Th17%	**4**	*1*	*8*	**5**	*3*	*7*	**5**	*4*	*11*	**7**	*3*	*10*	**8**	*5*	*11*	**9**	*6*	*14*	**<0.0001**
Th17 (×10^9/L)	**0,08**	*0*	*0.1*	**0.06**	*0*	*0.1*	**0.06**	*0*	*0.1*	**0.06**	*0*	*0.1*	**0.07**	*0.1*	*0.1*	**0.08**	*0.1*	*0.2*	**0.1678**
Th1 act %	**10**	*4*	*18*	**10**	*5*	*20*	**9**	*5*	*23*	**10**	*6*	*22*	**9**	*5*	*15*	**8**	*4*	*14*	**0.0816**
Th1 act (×10^9/L)	**0.01**	*0*	*0*	**0.02**	*0*	*0*	**0.01**	*0*	*0*	**0.01**	*0*	*0*	**0.01**	*0*	*0*	**0.01**	*0*	*0*	**0.3277**
Th2 act %	**6**	*2*	*15*	**5**	*2*	*12*	**4**	*2*	*15*	**7**	*2*	*12*	**6**	*3*	*9*	**6**	*3*	*10*	**0.2037**
Th2 act (×10^9/L)	**0.01**	*0*	*0*	**0.01**	*0*	*0*	**0.06**	*0*	*0*	**0.01**	*0*	*0*	**0.01**	*0*	*0*	**0.01**	*0*	*0*	**0.3621**
Th1–17 act %	**11**	*6*	*20*	**9**	*4*	*16*	**10**	*6*	*16*	**9**	*5*	*19*	**8**	*4*	*14*	**6**	*4*	*12*	**<0.0001**
Th1–17 act (×10^9/L)	**0.01**	*0*	*0*	**0.01**	*0*	*0*	**0.01**	*0*	*0*	**0.01**	*0*	*0*	**0.01**	*0*	*0*	**0.01**	*0*	*0*	**0.1689**
Th17 act %	**12**	*8*	*20*	**11**	*6*	*15*	**10**	*7*	*16*	**11**	*6*	*21*	**10**	*7*	*15*	**11**	*6*	*13*	**0.7628**
Th17 act (×10^9/L)	**0.01**	*0*	*0*	**0.01**	*0*	*0*	**0.01**	*0*	*0*	**0.01**	*0*	*0*	**0.01**	*0*	*0*	**0.01**	*0*	*0*	**0.5714**
RET %	**65**	*47*	*79*	**64**	*55*	*70*	**62**	*52*	*71*	**58**	*45*	*71*	**55**	*43*	*67*	**49**	*37*	*62*	**<0.0001**
RET (×10^9/L)	**1.44**	*0.6*	*2.7*	**0.8**	*0.6*	*1.6*	**0.81**	*0.3*	*1.3*	**0.59**	*0.3*	*1*	**0.51**	*0.3*	*0.8*	**0.52**	*0.3*	*0.7*	**<0.0001**
Treg %	**0.8**	*0.3*	*3.7*	**1.2**	*0.6*	*2.6*	**1.2**	*0.8*	*2.8*	**1.7**	*0.9*	*3.2*	**2**	*1*	*3.7*	**1.5**	*0.8*	*4.3*	**<0.0001**
Treg (×10^9/L)	**0.02**	*0*	*0.1*	**0.02**	*0*	*0*	**0.01**	*0*	*0*	**0.02**	*0*	*0*	**0.02**	*0*	*0*	**0.02**	*0*	*0*	**<0.0001**
CD19+ Transitional %	**17**	*8*	*27*	**15**	*10*	*30*	**14**	*9*	*24*	**18**	*9*	*24*	**13**	*8*	*21*	**9**	*5*	*15*	**<0.0001**
CD19+ Transitional (×10^9/L)	**0.16**	*0.5*	*0.4*	**0.07**	*0*	*0.1*	**0.06**	*0*	*0.1*	**0.04**	*0*	*0.1*	**0.03**	*0*	*0.1*	**0.02**	*0*	*0*	**<0.0001**
CD19+ Naive %	**87**	*71*	*94*	**74**	*63*	*86*	**76**	*65*	*86*	**70**	*51*	*85*	**76**	*64*	*84*	**76**	*65*	*88*	**<0.0001**
CD19+ Naive (×10^9/L)	**0.94**	*0.5*	*1.8*	**0.39**	*0.2*	*0.9*	**0.41**	*0.2*	*0.8*	**0.22**	*0.1*	*0.5*	**0.26**	*0.1*	*0.5*	**0.24**	*0.1*	*0.4*	**<0.0001**
CD19+ Preswitch Memory %	**6**	*2*	*10*	**7**	*4*	*13*	**9**	*5*	*16*	**9**	*5*	*17*	**7**	*4*	*14*	**8**	*4*	*12*	**<0,0001**
CD19+ Preswitch Memory (×10^9/L)	**0.06**	*0*	*0.1*	**0.05**	*0*	*0.1*	**0.05**	*0*	*0.1*	**0.03**	*0*	*0.1*	**0.02**	*0*	*0.1*	**0.02**	*0*	*0*	**<0.0001**
CD19+ Switch Memory %	**5**	*1*	*11*	**8**	*4*	*15*	**7**	*4*	*16*	**12**	*5*	*22*	**9**	*6*	*16*	**10**	*4*	*16*	**<0.0001**
CD19+ Switch Memory (×10^9/L)	**0.05**	*0*	*0.1*	**0.05**	*0*	*0.1*	**0.03**	*0*	*0.1*	**0.04**	*0*	*0.1*	**0.03**	*0*	*0.1*	**0.03**	*0*	*0.1*	**0.0022**
CD19+ Plasmablasts %	**12**	*6*	*46*	**9**	*3*	*19*	**9**	*5*	*17*	**9**	*4*	*21*	**6**	*2*	*11*	**8**	*4*	*15*	**<0.0001**
CD19+ Plasmablasts (×10^9/L)	**0.01**	*0*	*0*	**0**	*0*	*0*	**0**	*0*	*0*	**0**	*0*	*0*	**0**	*0*	*0*	**0**	*0*	*0*	**<0.0001**

CD19+ CD21low %	**3**	*1*	*7*	**3**	*2*	*6*	**4**	*3*	*10*	**4**	*2*	*10*	**3**	*2*	*7*	**4**	*2*	*6*	**0.64**
CD19+ CD21low (x10^9/L)	**0.03**	*0*	*0.1*	**0.02**	*0*	*0*	**0.02**	*0*	*0.1*	**0.02**	*0*	*0*	**0.01**	*0*	*0*	**0.01**	*0*	*0*	**<0.0001**
CD56+ Bright %	**6**	*1*	*12*	**5**	*2*	*16*	**5**	*2*	*10*	**4**	*1*	*10*	**4**	*2*	*8*	**4**	*2*	*8*	**0.1555**
CD56+ Bright (x10^9/L)	**0.02**	*0*	*0.1*	**0.02**	*0*	*0*	**0.02**	*0*	*0*	**0.01**	*0*	*0*	**0.01**	*0*	*0*	**0.01**	*0*	*0*	**<0.0001**
CD56+ Dim %	**88**	*75*	*93*	**86**	*72*	*91*	**88**	*77*	*91*	**89**	*80*	*93*	**90**	*83*	*93*	**91**	*79*	*95*	**0.0133**
CD56+ Dim (x10^9/L)	**0.48**	*0.1*	*1.2*	**0.33**	*0.1*	*0.8*	**0.31**	*0.1*	*0.5*	**0.31**	*0.1*	*0.5*	**0.29**	*0.2*	*0.5*	**0.29**	*0.1*	*0.7*	**0.1787**
Monocytes, Classical %	**81**	*66*	*92*	**89**	*68*	*94*	**88**	*82*	*96*	**90**	*73*	*94*	**91**	*82*	*94*	**93**	*85*	*95*	**0.0035**
Monocytes, Classical (x10^9/L)	**0.75**	*0.4*	*1*	**0.5**	*0.4*	*0.9*	**0.5**	*0.4*	*0.6*	**0.5**	*0.3*	*0.8*	**0.4**	*0.3*	*0.7*	**0.6**	*0.3*	*0.9*	**<0.0001**
Monocytes, Non-classical %	**6**	*2*	*12*	**3**	*2*	*9*	**3**	*1*	*9*	**3**	*1*	*7*	**3**	*1*	*5*	**2**	*1*	*4*	**0.285**
Monocytes, Non-classical (x10^9/L)	**0.05**	*0*	*0.1*	**0.03**	*0*	*0.1*	**0.02**	*0*	*0.1*	**0.01**	*0*	*0*	**0.01**	*0*	*0*	**0.01**	*0*	*0*	**0.0013**
Intermediate monocytes %	**3**	*1*	*10*	**3**	*1*	*9*	**4**	*1*	*8*	**2**	*1*	*8*	**2**	*1*	*5*	**2**	*1*	*4*	**0.0075**
Intermediate monocytes (x10^9/L)	**0.03**	*0*	*0*	**0.02**	*0*	*0.1*	**0.02**	*0*	*0.1*	**0.01**	*0*	*0*	**0.01**	*0*	*0*	**0.01**	*0*	*0*	**0.0001**
DC, Mieloid %	**69**	*41*	*87*	**65**	*54*	*83*	**68**	*32*	*94*	**70**	*40*	*83*	**60**	*44*	*85*	**72**	*41*	*83*	**0.7941**
DC, Plasmacitoid %	**18**	*5*	*45*	**20**	*9*	*40*	**23**	*4*	*55*	**20**	*8*	*42*	**26**	*10*	*45*	**22**	*13*	*51*	**0.5021**
TREC/100 ng DNA	**64**	*35*	*183*	**63**	*32*	*119*	**49**	*17*	*100*	**34**	*18*	*57*	**24**	*9*	*57*	**22**	*10*	*45*	**<0.0001**

counts of class switched and pre-switch B-cell populations decreased with age, with significant differences between groups. The percentage and absolute counts of plasmablasts gated in switched memory B cells decreased with increasing age, with significant differences between groups (Supporting Information Fig. S2F).

The relative population size of DC and NK cells remained stable in the different age groups. As to monocytes, the counts of both the classical and non-classical monocyte populations decreased with age (Supporting Information Fig. S2C).

Measurement of TRECs and Correlation with RTE and CD4+ Naive Populations and Age

Thymic function was evaluated by quantification of TRECs and RTE. As was expected, TREC values significantly decreased with age, but showed a large dispersion (Table 3). Correlations were investigated between TREC levels and RTE percentages between individual values. A better correlation was found between TREC levels and age (r^2 = 0.3127) than between RET and age (r^2 = 0.2801) (data not shown). The correlation between RTE and TREC values was slightly lower (r^2 = 0.2153) (Fig. 3A).

We also analyzed correlations of CD4+ naive T-cell percentages with TREC values and RTE. A better correlation was found between CD4+ naive T cells and RTE (r^2 = 0.4160, Fig. 3B) than between CD4+ naive T cells and TREC (r^2 = 0.3013) (Fig. 3C).

Application to the Diagnosis of Primary Immunodeficiencies

The distribution of naive and memory T cells is particularly relevant for the diagnosis of severe combined immunodeficiency (SCID) and its variants in infants—Omenn syndrome and leaky SCID—especially with the introduction of SCID screening in newborns (14,15). Additionally, RTE analysis can be used to evaluate and corroborate thymic function, as CD31 expression on naive CD45RA+CD4+T cells is associated with nascent T cells (16). Furthermore, B-cell subset phenotyping has been used to classify and assess the prognosis of patients with common variable immunodeficiency (CVID) (17).

We applied the above extended immunophenotyping panels to assess lymphocyte populations in the diagnosis of PID patients. Here, we report two illustrative examples of the diagnostic application of the panels. A 9-year-old boy had lymph node lymphoproliferation, splenomegaly, diarrhoea, dysgammaglobulinemia with hyper-IgM, and growth retardation. He had been initially diagnosed with CVID and had received immunoglobulin replacement and antibiotic prophylactic treatment. Extended immunophenotyping in this patient showed an increase compared with the age-matched reference values in the percentage of effector memory CD4+ T cells (62.6%) (14–18 years: 12%–44%), effector CD8+ T cells (39.4%) (14–18 years: 5%–20%),

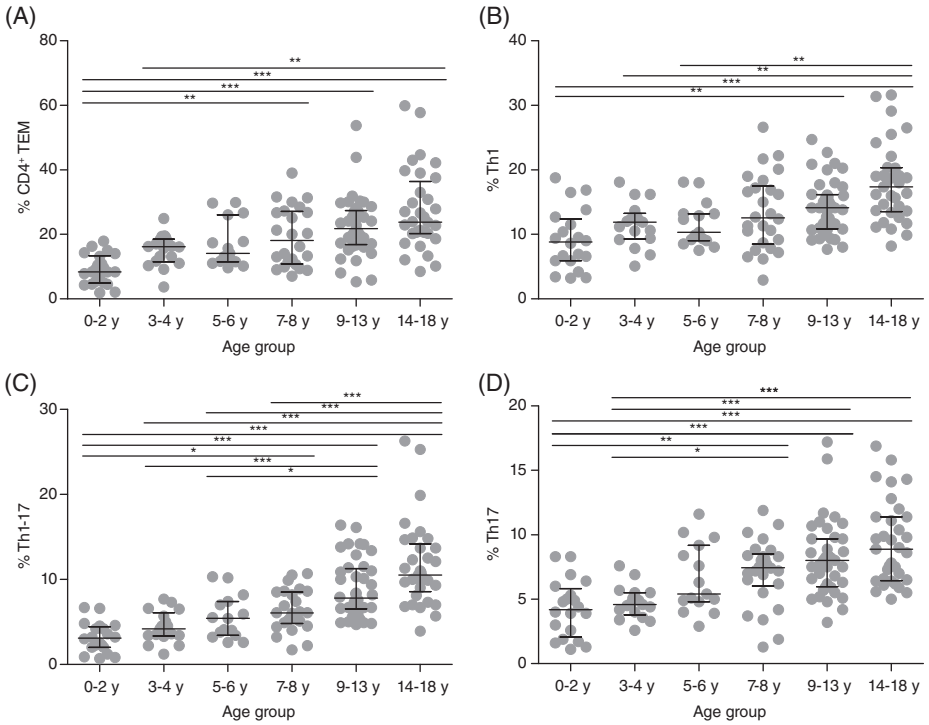

FIG. 1. Effector memory T cell and T helper subpopulations in healthy children by age group. The long line represents the median, short lines the interquartile range. **A**. Percentage of CD4+ effector memory T cells increased with age. **B**. Percentage of T helper 1 cells, **C**. T helper 1–17 cells and **D**. T helper 17 cells. *P* value indicates the significant differences between all groups. Significant differences between individual groups are indicated as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

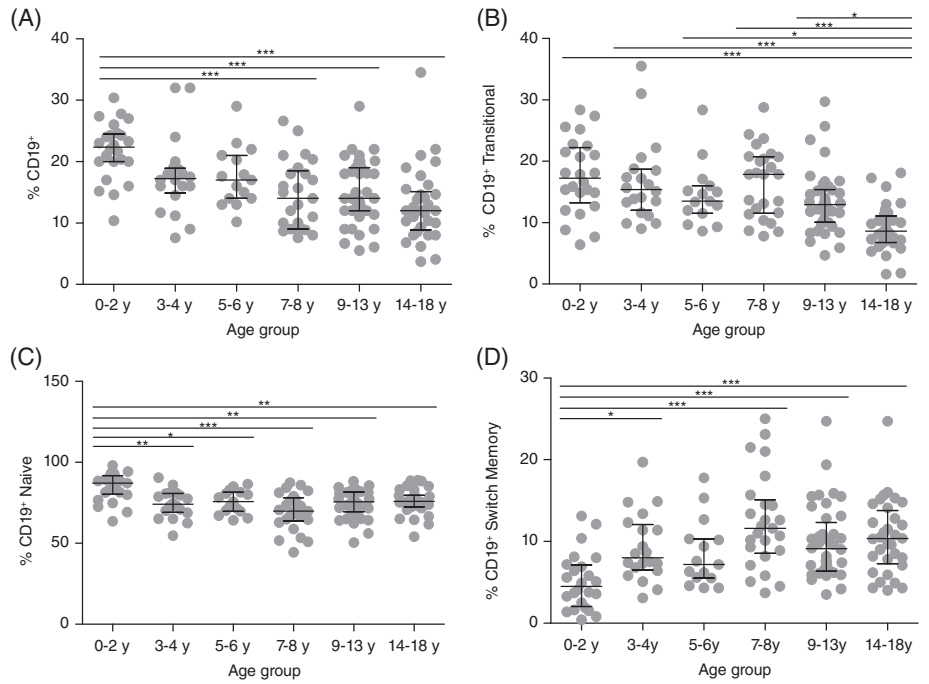

FIG. 2. B-cell subpopulations in healthy children by age group. The long line represents the median, short lines the interquartile range. **A**. Percentage of CD19+ cells decreased with age, as occurred in **B**. Percentage of CD19+ transitional cells and **C.** Percentage of CD19+ naive B cells. **D.** In contrast, the percentage of CD19+ switched memory cells increased with age. *P* value indicates the significant differences between all groups. Significant differences between individual groups are indicated as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

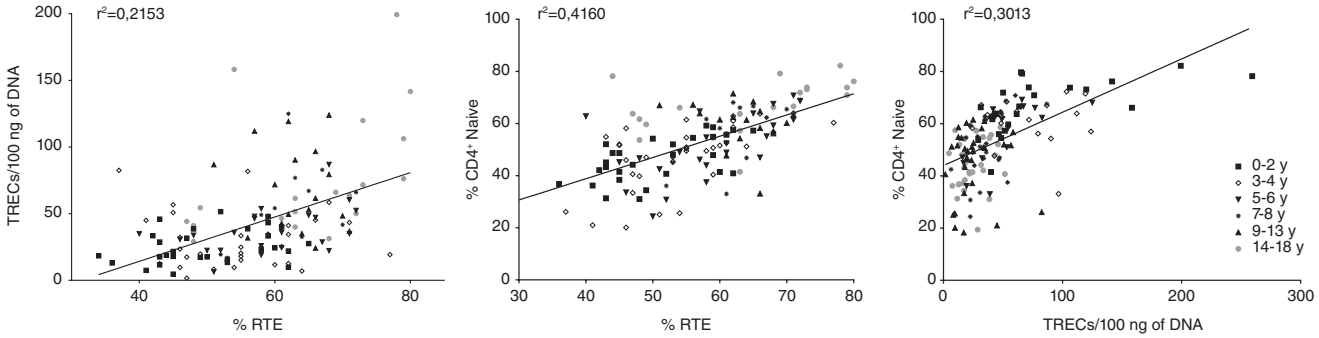

FIG. 3. Age-related correlation between TREC, RTEs, and CD4+ naive T cells. A.TREC level per 100 ng of DNA versus RTE percentage ($r^2 = 0.2153$). **B.** CD4 naive T cell percentage vs. RTE percentage ($r^2 = 0.4160$). **C.** CD4 naive T cell percentage versus TREC/100 ng of DNA ($r^2 = 0.3013$). Each symbol represents a healthy donor. Different types of symbols are used for different age groups.

and transitional CD19+ cells (70.2%) (14–18 years: 5%–15%) (Fig. 4A). The phenotype was suggestive of activated PI3K delta syndrome type 2 (APDS-2), which prompted a genetic analysis that showed a de novo mutation in the *PI3KR1* gene (c.1425+1G>A), affecting PI3 kinase. The mutation leads to skipping of exon 11 and loss of amino acid residues from 434 to 475 in the inter-SH2 protein domain (18). Thus, the extended immunophenotyping findings led to a diagnosis of APDS-2 and initiation of sirolimus treatment, with a good analytical and clinical response.

The second example is a 6-year-old girl with atopic dermatitis, recurrent otitis, and pneumonia, a pneumatocoele in the right upper lung lobe, and a laterocervical abscess caused by *Staphylococcus aureus*. Extended immunophenotyping showed a decrease in the patient's Th17 population (2.14%) (5–6 years: 4%–11%) compared with a healthy control (Fig. 4B). Based on

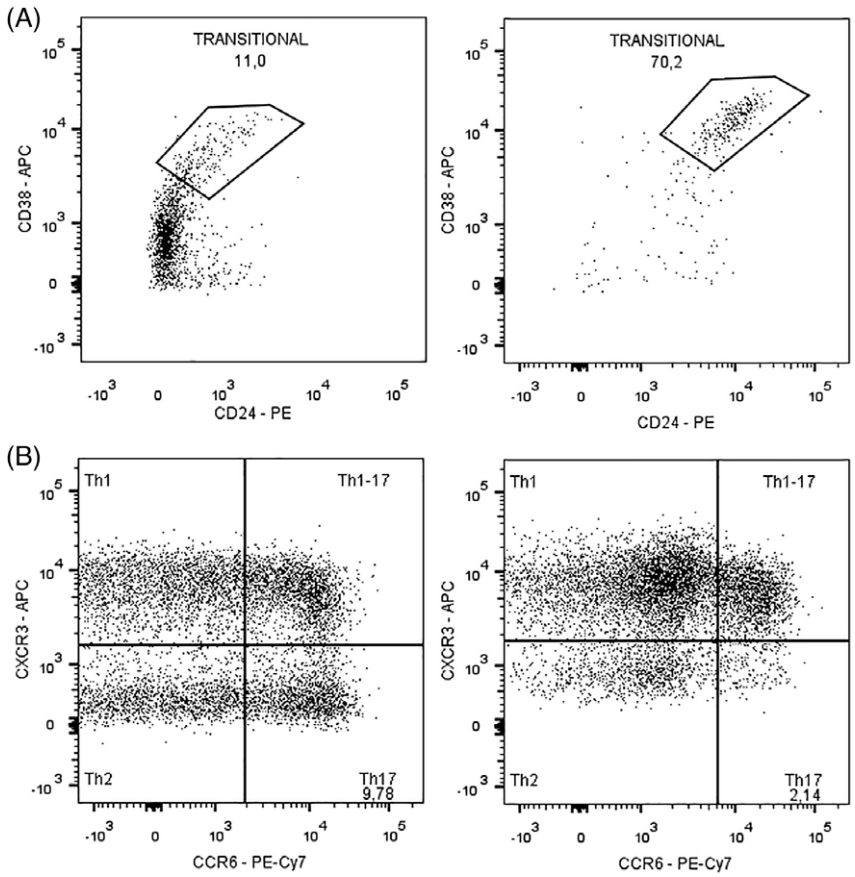

FIG. 4. Gating strategy examples. **A.** Gating strategy for an APDS-2 patient (right), where an increase in transitional CD19+ cells is seen compared with a healthy control (left). **B.** Gating strategy for a patient with hyper-IgE Syndrome (HIES) (right) due to STAT3 deficiency, showing a decrease in Th17 cells compared with a healthy control (left).

these data, the suspected diagnosis was hyper-IgE syndrome (HIES). Sanger sequencing detected a mutation in STAT3 (p.Val463del) with deletion of 3 nucleotides and loss of one amino acid at position 463, a mutation previously described in association with HIES (19).

DISCUSSION

Despite the impressive expansion of our knowledge of the immune system, the available tools to assess the immune status in a specific patient remain limited. In this scenario, extended phenotyping using multicolor flow cytometry is becoming increasingly more valuable as improved cytometers, additional fluorochromes, and standardized operating procedures are being introduced. However, although innate immunity seems to be stable overall, the cellular elements of the adaptive immune system measured by flow cytometry exhibit profound age-dependent changes (20). It is, therefore, mandatory to generate reliable reference values for each of the phenotypes, duly stratified by age. Here, we report the normal values for lymphocyte subpopulations obtained in a healthy population aged 1 month to 18 years that should be useful in the clinical setting, especially for the diagnosis of PID. Other studies have reported normal values in adults using similar approaches (21).

The composition of the major T-cell differentiation stages in the pediatric period was reported in a cross sectional study using CD45RA/CD27 for CD4 and CD45RA/CCR7/C27 for CD8 (22). The results obtained are similar to those found in our series: a gradual increase in effector/memory helper T cells with increasing age and a decrease in naïve T cells. Because of the progressive decline in the total lymphocyte population over time, the stable percentages of TCM and TEMRA resulted in an overall decrease in the absolute numbers of these populations. Naïve T cells exit from the thymus following maturation, are enriched by TRECs, and express the surface marker, CD31 (23). TRECs levels tend to decrease due to the age-related regression of the thymus. Thymic T-cell regeneration is quantitatively small throughout adult life as homeostatic proliferation is responsible for maintaining the size of the naïve T-cell compartment (24). There is little information in the literature on the evolution of TRECs, RTEs, and CD45RA naïve T cells with age within the pediatric period. In our study, the correlation of TRECs and RTEs was clear but lower than expected, likely due to the stabilization and high dispersion of TRECs values in the oldest age groups, in agreement with a report by Junge et al. who performed the same correlation according to age and in lymphopenic children (25).

Regarding the B-cell subpopulations, we found the same age-associated trends reported recently (7,26). We also studied the presence of CD19+CD21low cells, a distinct population of immature, polyclonal, unmutated B lymphocytes that express activation markers (27). Expansion of the CD21low B-cell population seems to be associated with a pathogenic role in several immune-mediated disorders, such as CVID and systemic lupus erythematosus. Data on the normal ranges of this cell population in different age groups is crucial to guide the diagnosis of these conditions (28). Concerning the monocyte, NK, and DC subsets, there were no differences between age groups in our population. Analysis of the monocyte and DC subsets did not show the variability found between cord blood and adult blood samples in populations with other environmental and genetic backgrounds (29).

The extensive age-stratified panel of reference values described here for most of the well-established lymphocyte subpopulations could be of value for the diagnosis and follow-up of the pediatric population. In our experience it has shown diagnostic value, as was seen in the two representative cases presented as examples. It is expected that translational research in immunology will continue to characterize lymphocyte populations and identify additional markers of activity, thereby providing better tools for the diagnosis and follow-up of patients with PID and other immune-mediated diseases.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

CLINICAL IMPLICATIONS OR KEY MESSAGES

This manuscript provides previously unavailable reference values for various leucocyte subsets in pediatric age groups to evaluate primary immunodeficiencies.

Extended immunophenotyping is a useful aid in the diagnosis of immune-mediated diseases.

AUTHORS CONTRIBUTION

M.G.P. performed the research design, data analysis, and writing of the manuscript.

D.A.S. participated in the research design and data analysis.

A.A.C., S.S.P, and S.B.S. participated in performing the research.

R.C. contributed in TRECs determination.

I. M. contributed with the hematological data.

M.G.P, A.M.N., P.S.P., C.F.J., M.H.G, R.P.B. reviewed the manuscript.

M.M.G. participated in conducting the study, and writing and reviewing the manuscript.

All the authors have read and approved the text submitted.

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