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Normative data for paediatric lymphocyte subsets: A pilot study from western India

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***Background & objectives*: Accurate diagnosis of immunodeficiencies requires a critical comparison of values with age-matched controls. In India, the existing reference values for rare lymphocyte subsets are currently not available and we rely on the data originating from other countries for the interpretation of the results. Furthermore, there is limited information on normal variation for these rare-subset parameters in Indian children. So, this study aimed to establish normative values for clinically important lymphocyte subsets in Indian children at different age groups.**

***Methods*: 148 children aged ≥16 yr were enrolled in this study. The study population included 61 per cent males and 39 per cent females and was divided into the following groups: cord blood (n=18), 0-6 months (n=9), 6-12 months (n=13), 1-2 yr (n=19), 2-5 yr (n=27), 5-10 yr (n=25) and 10-16 yr (n=37). The absolute and relative percentage of lymphocytes, T, B, natural killer cell, along with activated, naïve and memory subsets, was determined by flow cytometry.**

***Results*: Median values and the 10th and 90th percentiles were obtained for 34 lymphocyte sub-populations. The T and B naïve compartments showed a decreasing trend, whereas memory cells showed an increase with age. The activated T cell subset shows an increasing pattern up to one year and then declines gradually. Double negative T cells are relatively stable. TCRgd+T cell percentage increases with age.**

***Interpretation & conclusions*: This single-centre pilot study provides preliminary data that justifies the need for future large-scale multi centric studies to generate a reference range for interpreting extended immunophenotyping profiles in the paediatric age group, making it possible for clinicians to assess the immunological status in inborn errors of immunity, infectious and autoimmune diseases.**

Key words Children - flow cytometry - lymphocyte subset - pilot study - primary immunodeficiency

Flow cytometry functions as a rapid and efficacious technique for quantifying different leukocyte subsets, predominantly lymphocytes, along with the evaluation of expressed proteins. This method holds paramount significance in the diagnosis of inborn errors of immunity (IEI), as well as the assessment of immunological status in infectious and autoimmune diseases[1]. The age of presentation varies from infancy to late adulthood[2]. The patients with IEI present with recurrent infections or immune dysregulation[2]. There has been an exponential increase in the diagnosis of novel IEI in the last decade, leading to a better prognosis and management of these cases[2]. One of the initial steps in the diagnostic workup for patients with IEI includes peripheral blood immunophenotyping. Still, reliable age-wise reference values for rare lymphocyte subpopulations are limited[3,4].

Clinical decision-making of immune deficiencies relies on measurable deviations of the patient's values from normal, and this mainly depends on the availability of the reference ranges[4]. Flow cytometric age-related reference ranges of major lymphocyte subsets are available from various ethnicities[5-7]. Sub-characterization of these subsets is usually not performed as a part of routine standard phenotyping despite available knowledge regarding associations between lymphocyte subsets with certain disease states[8]. With the advent of a multicolour flow cytometer and a wide range of fluorescent-conjugated antibodies, sub-characterization of cells has become easier.

While data on the prevalence of IEI are lacking in India, it is speculated that India may have nearly one million patients with IEI[9,10]. Although reference ranges for lymphocytes are available for healthy adults[11-13] and children[14], there is a paucity of data for the rare subsets in the Indian population. This study presents paediatric age-matched reference values for lymphocyte subsets and some T and B cell subpopulations using the dual-platform method, which are essential for cell maturation and activations (both percentage and absolute count). This will provide a guideline for interpreting the immunophenotype as part of the diagnostic process for IEI and other immune diseases such as HIV and for immune monitoring in infectious diseases such as coronavirus disease (COVID)-19 and other autoimmune diseases.

Material & Methods

This pilot study was undertaken at the department of Paediatric Immunology and Leukocyte Biology, ICMR-National Institute of Immunohaematology (NIIH), Mumbai, India. The study was approved by the Institutional Ethics Committee of ICMR-NIIH, B J Wadia hospital for children and Nowrosjee Wadia Maternity Hospital.

Study population: A total of 148 healthy children, 0-16 years of age were recruited for the study between January 2018 to February 2021 (details in Supplementary Fig. 1). Cord blood (CB) samples from full-term healthy neonates and leftover ethylene diamine tetraacetic acid (EDTA) blood from healthy children, who underwent venipuncture for screening before benign surgical procedures without any evidence of infectious, immunologic or haematologic disorders, were used. For participants aged 10-16 yr, the sample was also collected through voluntary blood sampling at schools after procuring a written informed consent from their parents. Their overall health status was evaluated by detailed clinical history and physical examination.

Exclusion criteria were a history of previous sibling death, fever, rash, diarrhoea, recurrent cold, cough, blood transfusion and any medicine or steroids during the past one month or any history of chronic diseases such as tuberculosis, asthma, diabetes and atopic dermatitis.

For the CB sample, babies with normal birth weight and full term delivery were included. Family history of early sibling death, consanguinity of parents, newborn factors such as baby fever, jaundice, anaemia, low APGAR at birth and signs of sepsis were excluded from the study. Maternal factors such as illness in the mother during the last trimester, fever, history of diabetes, hypothyroidism, toxaemia, preterm, gestation prolonged rupture of membrane and recent illness in parents were excluded. The study population was 61 per cent males and 39 per cent females and was divided age wise into groups: CB (n=18), 0-6 months (n=9), 6-12 months (n=13), 1-2 yr (n=19), 2-5 yr (n=27), 5-10 yr (n=25) and10-16 yr (n=37).

Sample preparation: Whole blood (3 ml) was collected in EDTA vials by venipuncture and processed within 24-36 h of collection. The absolute lymphocyte number was determined from whole blood using Sysmex Haematology analyzer XS-800i (Sysmex Co., Cobe, Japan). The whole blood sample was used for flow cytometry analysis.

Immunophenotyping/multicolour staining & analysis: Immunophenotyping was carried out on 10-color

Navios Ex (Beckman Coulter, FL,USA) flowcytometer or 13- colour Dxflex (Beckman Coulter) flowcytometer. The acquisition was run until 50,000 CD3+T cells and 10,000 CD19+B cells were detected. Kaluza analysis software V2.1 (Beckman Coulter) was used for data analysis.

Multicolor flow cytometry was utilized for the identification of B, T, and natural killer (NK) cells along with their distinct subsets (details in Supplementary Table I). Singlet gating strategy was applied to eliminate aggregates, and scatter gates were defined specifically for lymphocytes. The lymphocyte population was discerned from the sample by employing forward scatter and side scatter (SS) gating. Furthermore, this lymphocyte population, characterized by low forward and SS values, was evaluated for its purity through CD45 positivity analysis. Immunophenotype markers identified subsequent lymphocyte subpopulations. At certain fluorochrome, dual markers were combined due to their mutually exclusive presentation, *i.e*. one B cell and another T cell (*e.g*. CD8 with IgD and CD19 with TCRgd), while CD16 and CD56 were used to detect NK cells[1].

To assess the cell composition, the wash-stain-lyse-wash method was used[15]. Briefly, 200 µl of whole blood was washed twice with phosphate-buffered saline (PBS). The cells were incubated in dark for 20 min at room temperature with a mixture of optimally titrated monoclonal antibodies (details in Supplementary Table II), followed by lysis using Optilyse C™ (Beckman coulter) for 10 min. After washing once with PBS, the cells were acquired on DxFlex or Navios Ex (Beckman Coulter) flow cytometer and analyzed using Kaluza v2.1 data analysis software (Beckman Coulter). The determination of absolute subset cell counts was achieved through a dual platform approach involving the multiplication of the subset proportion acquired *via* flow cytometry and the absolute lymphocyte count as assessed using a 5 part haematology analyzer (Sysmex Co.,Cobe, Japan).

Quality control: Machine daily quality check was ensured using quality control beads for DxFlex (Beckman coulter) using Cytoflex beads and Navios Ex (Beckman Coulter) using the Flow-Check fluorospheres. Quality control was run according to the manufacturer's instructions and standard laboratory protocol. Levey-Jennings chart was prepared using Kaluza analysis software and half peak CV for all parameters were plotted, which was within the acceptable limits (±2 standard deviation), ensuring reliability and accuracy of the machine.

Statistical analysis: The number of participants in different age groups varied from nine to 37 (n=148). Data analysis used the GraphPad Prism software, version 9.1 (GraphPad Prism, CA, USA), RStudio- V1.4 (R Core Team, Vienna, Austria) and Microsoft Excel (Microsoft Office 2010, USA). For each cell population, the normal range was defined based on the median, as well as the 10^{th} and 90^{th} percentiles of the cell frequencies. Each variable was analysed in both absolute number as well as percentage. Differences between study groups (age) were assessed by the Kruskal-Wallis test followed by Dunn's multivariate comparison. The $P<0.05$ was considered significant. An unpaired t test was used to compare subsets of helper and cytotoxic T (Tc) cells to test the difference between CD62L and CD27.

Results

An immunophenotyping platform was established to explore the variation in immune system composition. This platform utilizes flow cytometry to quantitatively assess over 34 discrete immunological attributes, with specific emphasis on subsets within the adaptive immune system The median along with 10^{th}-90^{th} percentiles of absolute and relative size for different age groups are shown in Tables I and II, respectively. Significant variation was observed among different age groups. Figures 1 and 2 show the gating strategy for lymphocyte sub-populations. Using this gating strategy, age-related reference values for relative percentage and absolute lymphocyte subset counts of 148 healthy individuals among seven different age groups were calculated. The values are represented as box plots with median, p10, p25, p75 and p90 percentile in Figures 3 and 4.

The presented data has unveiled noteworthy trends in the lymphocyte and T-lymphocyte profiles across age groups. During early infancy, there is a conspicuous elevation in the absolute counts of total lymphocytes and T-lymphocytes, which subsequently diminish with advancing age. Notably, the proportion of T cells while varying slightly with a median range spanning from 62 to 73 per cent showed a stable pattern over time. Furthermore, specific T cell subsets such as Th and Tc cells remained relatively constant, exhibiting medians within the range of 35 to 48 per cent for Th cells and 17.8 to 27 per cent for Tc cells.

Table I. Median and 10-90th percentile of lymphocyte and its subpopulation absolute number (/ul blood) by age group

Absolute count/ age	CB (n=18)	0-6 months (n=9)	6-12 months (n=13)	1-2 yr (n=19)	2-5 yr (n=27)	5-10 yr (n=25)	10-16 yr (n=37)
Lymphocytes***	3627	6013[y,x]	6029[u]	5210[r,t,q]	4226[o]	3108	3087
	2809-5917	3744-8637	3537-8294	3182-7410	3048-6752	2258-4781	2038-4113
NK cell	250	338	297	342	325	258	238
	69-1412	220-779	84-1237	144-565	130-636	90-679	110-437
T cell***	2423	4321	3952[u,t]	3642[r,q]	2744[o]	2300	2291
	1889-4764	2617-5380	2252-5788	2105-5460	2087-4899	1639-3388	1390-3008
Th cell***	1637[δ,%]	2802[y,x]	2274[u,t]	2090[r,q]	1463[o]	1109	1168
	1060-3558	1336-3327	1207-4013	1154-3460	1001-2535	805-1855	659-1547
Tc cell***	707[#,$,^]	1010	1315[t]	1185[q]	1057	856	841
	451-1346	618-1778	548-1930	781-1692	562-1809	508-1257	471-1242
Th Naive	1445[δ,%]	2619[>,y,x]	1797[u,t]	1552[r,q]	1057	825	644
cell (CD27)***	1001-3329	993-3086	758-2992	816-2509	711-1844	416-1462	325-1208
Th RTE***	1297[δ,%]	1905[y,x]	1625[u,t]	1375[r,q]	806[o]	570	459
	842-2911	766-2563	593-2977	701-2388	522-1456	342-1126	227-860
CM Th	128[#,$,^,δ,%]	285	293	351	300	283	337
cell (CD27)***	57-290	195-363	196-605	195-753	215-580	151-420	172-511
EM Th	1[@,$,^,δ,%]	35	25[t]	37[q]	38[o]	49	76
cell (CD27)***	0-4	7-84	11-95	9-126	17-87	23-148	49-155
TEMRA Th	0[$,^,δ,%]	14[x]	9[t]	8	4	39	21
cell (CD27)***	0-1	1-56	1-157	1-218	1-29	4-131	3-103
Tc Naive	624	869[x]	773	757[q]	790[p,o]	537	414
cell (CD27)***	418-1206	434-1135	410-1223	437-1185	456-1293	306-844	244-829
Tc RTE***	658	816	899[t]	779[q]	753[o]	615.5	471
	435-1229	391-1088	447-1379	516-1289	422-1265	312-870	273-855
CM Tc	58[@,$,^,δ,%]	167	133	161	110	122	142
cell (CD27)***	22-115	73-320	38-417	46-352	53-349	40-242	51-245
EM Tc	0[@,#,$,^,δ,%]	31	23	19	12	16	26
cell (CD27)***	0-1	1-180	1-223	3-76	2-114	5-117	9-103
TEMRA Tc	0[@,#,$,^,δ,%]	72	112	133	65	131	108
cell (CD27)***	0-4	1-346	3-962	8-306	5-424	21-301	25-378
RTE on Th	1251[δ,%]	1473[y,x]	1564[u,t]	1277[r,q]	749[o]	495	445
Naive***	822-2720	776-2186	582-2605	676-1879	476-1314	281.5-1022	204-812
RTE on Tc	646[%]	744[x]	686[t]	703[q]	698[p]	468.5	385
Naive***	412-1136	345-908	326-1122	438-1126	379-1080	273-782	186-596
B cell***	628[@,#,$]	1912[y,x]	1405[u,t]	1124[r,q]	815[p,o]	440	556
	255-972	240-2636	715-2531	718-2007	510-1662	286-924	273-813
Total memory B	15[#,$,^,δ,%]	81[>,z]	134	170[q]	168	106	116
cell***	7-38	13-139	43-406	66-267	64-253	46-225	26-175
Class switch	0[#,$,^,δ,%]	10	37	78	51	41	53
memory B cell***	0-1	2-70	12-331	25-160	25-106	15-90	11-113

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Absolute count/ age	CB (n=18)	0-6 months (n=9)	6-12 months (n=13)	1-2 yr (n=19)	2-5 yr (n=27)	5-10 yr (n=25)	10-16 yr (n=37)	
Unswitched	14[^]	39	33[t]	38[q]	35[o]	23	13	
memory B cell[***]	7-32	06-69	13-161	6-99	7-93	5-69	02-31	
IgM only	0[#,$,^,δ,%]	4	6	6	5	3	5	
memory B cell[***]	0-0	0-5	3-17	2-21	01-19	1-11	1-17	
Naive B cells[***]	595	1701[y,x]	1254[u,t]	963[r,q]	659[o]	314	391	
	240-923	222-2512	638-2137	547-1811	342-1428	193-764	195-658	
Pre-GC B	575[δ,%]	786[y,x]	542[u,t]	542 [r,q]	379[o]	170	75	
cells[***]	238-903	132-2464	410-1847	69-906	98-908	52-554	23-407	
Double negative	0	1	1	1	1	1	1	
B cell	0-1	0-2	0-2	0-1	0-1	0-2	0-1	
CD4-CD8-DNT	72[#,$,^,δ,%]	129	215	187	232	148	165	
on Lymphs[***]	30-168	77-355	68-288	116-356	112-402	111-406	81-375	
CD4+CD8+DPT	45	80[y,x]	53	35	37	24	25	
on Lymphs[***]	15-113	28-461	11-146	17-114	13-116	15-57	13-80	
DNT gd-ab+T[***]	26[#,$,^]	51	59	88[r,q]	85[p,o]	45	40	
	14-56	17-149	30-105	42-137	48-125	30-90	26-65	
DNT gd+T[***]	74.5[#,$,^,δ,%]	121	253	209	234	182	165	
	25-167	76-205	66-441	128-348	126-470	84-467	67-359	
TCR gd-ab+T[***]	2370	4143[y,x]	3617[u,t]	3204[r,q]	2556[o]	2086	2004	
	1819-4662	2226-4835	2157-5516	1946-4883	1875-4537	1484-2923	1297-2694	
TCR gd+T[***]	68[@,#,$,^,δ,%]	191	246	206	241	189	189	
	26-174	119-545	73-558	135-340	133-406	97-390	64-353	
NKT	259	338	444	359	380[o]	361	224	
	141-622	145-841	134-1328	173-924	89-1180	203-778	71-516	
HLADR+Th	7[@,#,$,^,δ,%]	45	53	35	26.5	31	38.5	
cellv	2-24	32-120	22-76	16-117	17-97	17-64	13-67	
HLADR+Tc	3[@,#,$,^,δ,%]	142	101	108	41.5	50	50	
cell[***]	1-8	13-295	14-263	21-209	14-195	20-124	9-139	
HLADR+T	14.5[@,#,$,^,δ,%]	281	194	182	85	90	106	
cell[***]	6-34	62-585	57-403	48-391	38-287	55-201	38-200	
HLADR+NK	19[@,$]	66	39	53	39	36	30	
cell[*]	3-450	19-199	22-89	20-102	16-118	13-92	13-50	
HLADR+45RA	1[#,$,^,]	4	4	4	4	2	2	
of DNTgd-T[***]	0-3	0-6	02-13	0-7	0-7	1-5	1-3	
Th Naive	1503[δ,%]	2177[z,y,x]	1811[u,t]	1578[r,q]	957[o]	632	564	***
cell (CD62L)[***]	944-3371	875-2896	793-3181	791-2812	627-1570	388-1268	285-995	
CM Th	114[@,#,$,^,δ,%]	337	233	303	192	199	268	
cell (CD62L)[***]	28-178	50-465	100-468	146-494	104-477	83-342	152-447	
EM Th	40.5[#,$,^,δ,%]	129	142	154	212	153	201	
cell (CD62L)[***]	12-102	0-474	28-312	85-341	66-337	74-256	77-314	
TEMRA Th	56.5	25	49	66	30	72.5[n]	17	
cell (CD62L)[*]	14-146	2-418	11-381	7-460	1-324	8-236	7-72	

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Absolute count/ age	CB (n=18)	0-6 months (n=9)	6-12 months (n=13)	1-2 yr (n=19)	2-5 yr (n=27)	5-10 yr (n=25)	10-16 yr (n=37)
Tc Naive	656[%]	823[x]	754[t]	821[q]	791[o]	633.5	470
cell (CD62L)	419-1146	369-1169	374-1307	506-1243	405-1233	301-991	230-653
CM Tc	28[$,^,%]	78	50	92	97	46	93
cell (CD62L)	6-79	29-393	17-236	28-309	24-238	10-253	18-218
EM Tc	2[#,$,^,δ,%]	24	60	63	34	101	69
cell (CD62L)	0-13	7-198	11-364	12-269	12-327	11-297	22-242
TEMRA Tc	12[#,$,%]	52	84	113	76	29	136
cell (CD62L)	0-113	8-279	18-708	27-284	6-362	6-188	10-355

P[*]<0.05,[**]<0.01,[***]<0.001. Difference in intergroup are represented as: [@]CB *vs*. 0-6 months; [#]CB *vs*. 6-12 months; [$]CB *vs*. 1-2 yr; [^]CB *vs*. 2-5 yr; [δ]CB *vs*. 5-10 yr; [%]CB *vs*. 10-16 yr; [¨]0-6 months *vs*. 6-12 months; >0-6 months *vs*. 1-2 yr; [z]0-6 months *vs*. 2-5 yr; [y]0-6 months *vs*. 5-10 yr; [x]0-6 months *vs*. 10-16 yr; [w]6-12 months *vs*. 1-2 yr; [v]6-12 months *vs*. 2-5 yr; [u]6-12 months *vs*. 5-10 yr; [t]6-12 months *vs*. 10-16 yr; [s]1-2 yr *vs*. 2-5 yr; [r]1-2 yr *vs*. 5-10 yr; [q]1-2 yr *vs*. 10-16 yr; [p]2-5 yr *vs*. 5-10 yr; [o]2-5 yr *vs*. 10-16 yr; [n]5-10 yr *vs*. 10-16 yr. RTE, recent thymic emigrant; CM, central memory; EM, effector memory; TEMRA, terminally differentiated effector memory re-expressing CD45RA+; Tc, T cytotoxic; Th, T helper; CB, cord blood

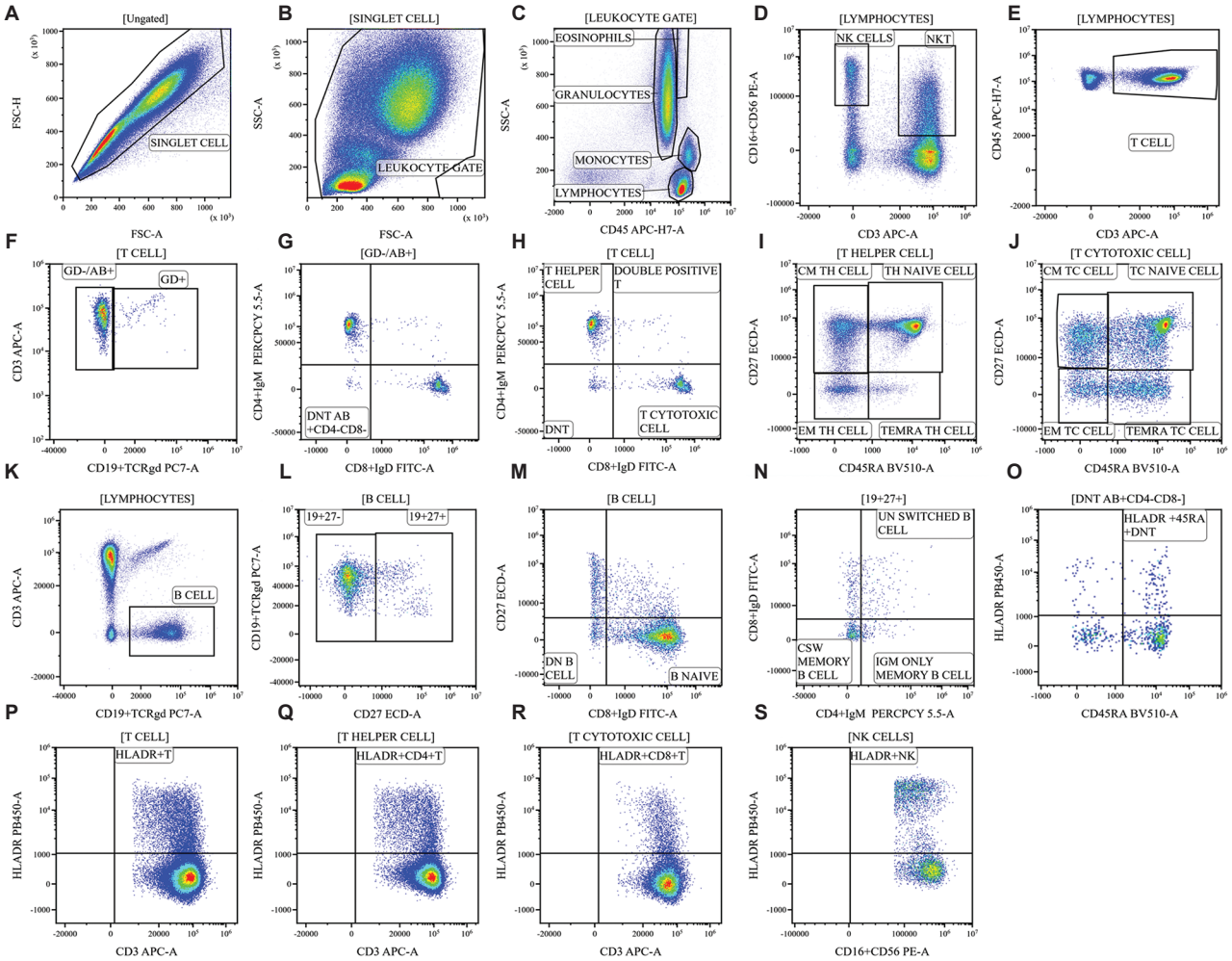

Fig. 1. Stepwise gating strategy for lymphocytes, general lymphocyte subpopulation, T, B, NK and activated cell subpopulations. (**A**) FSC-H vs. FSC-A plot was used to remove doublets and cell debris. (**B**) Singlet cells were further plotted against SSC-A vs. FSC-A. (**C**) SSC-A vs. CD45 was used to gate lymphocytes, based on low forward/side scatter and bright CD45. (**D**) Density plot demonstrating NK cells (CD3-CD16/CD56+) and NKT cells (CD3+ CD16/CD56+). (**E**) T cells were gated using CD45 and CD3. (**F-J**) T cell populations were distinguished depending on CD4, CD8, CD27, CD45RA and TCRgd expression. (**K-N**) B cells were gated as CD19+CD3- and the subpopulations were distinguished depending on IgD, IgM and CD27 expression. (**O-S**) HLA-DR was used to access the activation status on DNTs, T, Tc, Th, NK cells respectively. The names written above the plot [name] represent the parent gate. NK, natural killer

Table II. Median and 10-90th percentile of lymphocyte and its subpopulation relative percentage by age group

Percentage/age	CB (n=18)	0-6 months (n=9)	6-12 months (n=13)	1-2 yr (n=19)	2-5 yr (n=27)	5-10 yr (n=25)	10-16 yr (n=37)
Lymphocytes[a***]	39.3[@,#,$]	62.5[z,y,x]	58.8[u,t]	57[s,r,q]	43.4	38.7	39
	24.2-48.4	41.6-73	39.1-69.4	49.8-64.1	31.3-61.	24.5-49.0	27.5-48.2
NK cell[b]	6.3	5.6	5.2	6.7	7.2	8.8	8.3
	2.7-26.0	3.9-9.0	2.3-20.8	2.98-11.2	2.92-14.0	3.05-20.4	4.6-12.1
T cell[b*]	68.4	62.3	63.7	65.3	68.6	72.7	70.9
	55.9-85.7	52.1-86.3	49.5-75.9	57.7-74.3	57.1-75.5	60.5-78.8	61.4-79.6
Th cell[b***]	48.8[^,δ,%]	39.3	42.6	37.4	34.5	36.5	35.1
	31.5-61.3	35.3-63.5	21.0-51.2	31.5-49.4	28.7-42.2	27.0-47.1	27.8-44.6
Tc cell[b***]	17.8[^,δ,%]	18.4[z,y,x]	19.3[t]	23.3[q]	26.9	26.5	27.3
	12.7-27.8	13.1-22.3	13.7-28.5	17.8-26	17.6-34.8	19.2-36.5	20.7-36.7
Th Naïve	93.3[$,^,δ,%]	86.96[y,x]	83.9[t]	78[q]	75.7	71	61.6
cell (CD27)[c***]	84.5-96.6	74.3-92.8	57.2-90.3	62.3-86.8	63.9-81.0	51.7-82.2	39.9-76.8
Th RTE[c***]	76.7[^,δ,%]	68[x]	71.1[t]	61.4[q]	58.1[o]	52.3	43.6
	65.1-85.1	57.4-78.1	49.1-77.2	48.9-72	43.9-67.7	42.2-64.2	31.7-59.8
CM Th	6.5[$,^,δ,%]	11.4[z,y,x]	13.6[t]	19	20.7	24.3	28.8
cell (CD27)[c***]	3.03-15.2	6.8-19.8	8.9-26.8	11.7-27.3	17.4-29.2	15.2-35.8	17.2-49.2
EM Th	0.03[$,^,δ,%]	1.2[x]	1.1[t]	1.7[q]	2.7[o]	3.7	6.7
cell (CD27)[c***]	0.01-0.2	0.2-3.6	0.4-6.9	0.4-8.0	1.2-5.4	1.9-13.9	4.1-15.3
TEMRA Th	0[@,#,$,^,δ,%]	0.44	0.37	0.43	0.27[p,o]	2.7	2.14
cell (CD27)[***]	0-0.1	0.02-2.4	0.02-11.5	0.06-10.6	0.05-2.3	0.2-11.4	0.2-6.9
Tc Naïve	91.1[#,$,^,δ,%]	70.3	71.5	66.7	76.4	66.2	57.5
cell (CD27)[d***]	87.1-96.9	60.4-92.7	35.2-91.7	48.5-88.9	53.7-90.9	43.2-77	36.4-80.6
Tc RTE[d***]	94.9[#,$,^]	70.96	71.8	66.7	74.5[o]	68.0	63.3
	90.2-98.3	54.4-94.7	40.5-89.0	50.7-81.3	56.4-88.5	52.7-75.2	43.4-77.5
CM Tc	9.0[$,δ,%]	14.6	10.5	16.2	12.7	14.9	18.34
cell (CD27)[d*]	3.1-13.3	7.1-26.2	6.6-29.9	4.7-31.6	4.20-22.8	4.9-28.1	9.8-30.4
EM Tc	0.01[@,#,$,^,δ,%]	2.7	1.5	1.6	1.2	1.9	4.1
cell (CD27)[d***]	0-0.2	0.1-11.7	0.1-10.8	0.3-7.9	0.3-8.6	0.5-12.8	1-9.2
TEMRA Tc	0.04[#,$,^,*,%]	7.5	12.0	12.6	5.9	13.8	17
cell (CD27)[d***]	0-0.7	0.1-19.5	0.2-47.1	1.10-26.7	0.8-30.1	3.0-36.5	3.1-35.7
RTE on Th	71.2[^,δ,%]	60.2[x]	64.5[u,t]	58.9[r,q]	50.8	45.1	44.9
Naïve[c***]	58.6-78.9	50.5-69.6	48.2-73.3	45.4-68.9	39.9-63.3	30.7-57.4	30.3-57.1
RTE on Tc	92.7[@,#,$,^,δ,%]	59.2	60.4	59.1	65.5[o]	58.3	47.32
Naïve[d***]	83.8-96.1	49.5-79.8	26.9-84.1	38.2-78.4	43.7-80.4	35.7-68.6	32.1-66.2
B cell[b***]	14.2[@,$]	25.5[y]	23.3[u]	23.8[r,q]	21.6	16.6	17.9
	8.1-28.4	4.6-35.9	12.4-37.8	16-36.9	12.3-34.1	10.5-26.2	10.7-26.3
Total memory B	2.5[$,^,δ,%]	5.3[z,y,x]	10.1[u,t]	14.3	16.6	25.1	19.4
cell[c***]	1.7-5.4	1.3-16.8	3.8-19.6	7.3-21.8	8.7-30.0	10.6-35.2	7.2-33.7
Class switch	0.03[$,^,δ,%]	0.9[y,x]	3.2[t]	6	5.9	8.9	9.8
memory B cell[c***]	0-0.2	0.1-9.05	0.89-16	2.96-9.7	3.8-10.7	4.1-15.1	3.8-7.5

Contd..

Percentage/age	CB (n=18)	0-6 months (n=9)	6-12 months (n=13)	1-2 yr (n=19)	2-5 yr (n=27)	5-10 yr (n=25)	10-16 yr (n=37)
Unswitched	2.3	2.4	2.5	2.7	5	4.8	2.6
memory B cell[e*]	1.5-4.96	0.36-7.34	0.9-8.99	0.6-9	1.0-10.15	0.9-14.94	0.7-6.4
IgM only	0.02[#,$,^,δ,%]	0.19[x]	0.42	0.49	0.68	0.53	1.04
memory B cell[e***]	0-0.06	0-0.61	0.23-1.13	0.12-1.9	0.09-1.93	0.12-2.42	0.16-3.06
Naïve B cells[e***]	94.7[$,^,δ,%]	92.7[z,y,x]	86.8[u,t]	82.4	79.7	70.4	75.9
	92.6-96.4	79.9-96.6	74.1-92.7	73.6-90.2	67.1-88	59.8-82.9	59.8-89.6
Pre-GC B	92.6[#,$,^,δ,%]	71.2[x]	51.4[t]	49.4	47.5	32.3	14.9
cells[e***]	88.2-96.0	16.5-93.5	28.6-82.8	6.6-80.9	12.9-75.2	11.1-74.6	4.4-71.3
Double negative	1.44[δ,%]	2.04	3.28	2.69	2.7	4.21	4.54
B cell[e***]	0.7-3.99	0.99-5.44	0.63-7.27	1.02-7.07	1.01-4.60	1.3-8.3	1.6-8.3
DNT on Lymphs[b]	1.7[#,$,^,δ,%]	2.1[z,y,x]	3[v,u,t]	3.8	5.35	5.31	5.26
	0.91-3.37	1.02-9.47	1.5-4.5	3.3-5.6	3.1-8.2	3.3-9.9	3.1-10.9
DPT on Lymphs[b]	1.15	1.67	1.06	0.81	0.85	0.72	0.79
	0.44-3.16	0.54-5.34	0.23-2.83	0.31-2.01	0.34-2.42	0.498-1.766	0.43-2.38
DNTgd-ab+T[f***]	0.68[$,^,δ,%]	0.88>[z]	1.15[v]	1.72	1.85[o]	1.45	1.32
	0.44-1.44	0.24-1.85	0.57-1.62	0.85-2.4	1.19-2.7	1.13-2.27	0.95-2.02
DNT gd+T[f***]	1.68[$,^,δ,%]	2.36[z,y,x]	3.54	3.93	5.77	5.66	5.27
	0.65-3.14	1.4-12.01	1.58-6.82	2.82-8.03	2.86-9.73	2.88-12.45	2.45-10.86
TCRgd-ab+T[f***]	96.9[$,^,δ,%]	95.2	94	93.9	90.8	91.5	90.1
	94.9-98.7	85.0-97.4	83.5-97.2	88.5-95.1	86.2-95.3	84.99-96.3	83.8-95.8
TCRgd+T[f***]	2.6[#,$,^,δ,%]	4.8	5.3	6	8	8.5	9.1
	0.9-4.0	2.6-14.6	3.0-15.8	4.3-11.6	4.6-13.3	3.7-14.9	3.6-13.9
NKT[b]	7.1	6	7.6	7.6	10	11.2	7.4
	4.2-14.1	2.8-10.4	2.2-26	3.3-19.1	2.5-21.8	6.6-27	2.2-19.3
HLADR+Th	0.3[@,#,$,^,δ,%]	2	2.3	1.6	1.8	2.2	3.8
cell[c***]	0.2-1.5	1.2-5	0.7-5.7	0.8-7.5	1.2-7.3	1.3-6.7	1.4-5.9
HLADR+Tc	0.45[@,#,$,^,δ,%]	14.3	7.2	7.1	3.5	5.2	6.4
cell[d***]	0.2-1	1.2-19.3	2-16.2	1.9-22.1	1.6-17.0	2.8-16.8	2.3-18.7
HLADR+T	0.49[@,#,$,^,δ,%]	6.35	4.9	3.95	2.6	3.5	4.5
cell[f***]	0.3-1.4	1.4-10.9	1.4-10.1	1.3-14.3	1.4-8.1	2.1-10.0	2.1-10.2
HLADR+NK	6.4[@,$,^,δ,%]	22.2	15.2	13.7	13.4	13.8	12.9
cell[g]	2.2-20.5	4.3-38.6	4.6-44	5.8-32.1	7.1-21.5	6.8-29.7	7.7-32.8
HLADR+45RA	3	6.2	7.6	3.8	4.6	3.9	4.2
OF DNTgd-Th	0.4-10.6	2.6-10.9	2.1-14.2	0.9-7.8	0.5-9.3	1.4-7.9	1.2-12.1
Th Naïve	88.3[$,^,δ,%]	80.8[y,x]	78.0[u,t]	71.4[q]	65[o]	54.5	55.2
cell (CD62L)[c***]	80-95.5	65.5-87.1	64.7-83.7	59.5-82.8	54.8-81.3	41.8-79.7	33.8-70.7
CM Th	5.7[$,^,δ,%]	14.3[x]	11.8[t]	14.3[q]	15.4[o]	17.9	25
cell (CD62L)[c***]	1.7-11.7	2.1-19.4	3.5-19.8	7.6-22.2	6.3-23.1	7.4-32.3	16-33.1
EM Th	2.4[^,*,%]	4.6[y,x]	7.6[u,t]	7.9[q]	12.5	14.9	17.5
cell (CD62L)[c***]	0.3-5.3	0-20.1	1.20-10.8	2.9-15.9	4.9-18.7	5.2-22.9	9.2-30.6
TEMRA Th	3.2	1.1	3.2	2.9	2.2	6.7[n]	1.7
cell (CD62L)[c*]	0.9-6.9	0.1-13.5	0.6-11.2	0.6-16.6	0.1-17.9	0.9-20.8	0.7-8.06

Contd..

Percentage/age	CB (n=18)	0-6 months (n=9)	6-12 months (n=13)	1-2 yr (n=19)	2-5 yr (n=27)	5-10 yr (n=25)	10-16 yr (n=37)
Tc Naïve	93.8[@,#,$,^,*,%]	67.3	62	70.3	74.8[o]	71.2[n]	53.4
cell (CD62L)[d***]	84.5-96.8	59.7-85.1	31.9-86.1	44.8-88.1	53.7-85.8	45.5-87	37-72.9
CM Tc	3.4[^,%]	8.2	4.5	7.7	8.2	5.1	7
cell (CD62L)[d**]	1.1-8.1	3.0-28	1.2-15.3	2.9-24.8	2.1-19.7	1.1-29.6	3.0-29.7
EM Tc	0.3[#,$,^,*,%]	2	5.2	6.5	3.3	11.9	11.5
cell (CD62L)[d***]	0-1.6	0.7-29.7	1.3-25.8	0.95-22.6	1.2-21.3	1.3-29.1	2.3-26.5
TEMRA Tc	2[#,*,%]	5.5	8.8	9.7	5.5[o]	3.6[n]	20.2
cell (CD62L)[d***]	0-9.3	0.6-29.4	1.8-57.3	1.9-26.5	0.63-24.9	0.7-22.7	2.7-37.2
gd+T of	2[^,*,%]	3	4	4	6	6	6
Lymphs[b***]	0.9-3.1	2-10	1.4-8.4	3-5.4	3-9	2.6-10.4	2.8-10.2
CD4/CD8 ratio[***]	2.49[^,*,%]	2.74[z,y,x]	2	2	1	1	1
	1.1-3.8	2-4	1-3.6	1-3	1-2	1-2.4	1-2

P[*]<0.05, [**]<0.01, [***]<0.001. Difference in intergroup are represented as: [@]CB *vs*. 0-6 months; [#]CB *vs*. 6-12 months; [$]CB *vs*. 1-2 yr; [^]CB *vs*. 2-5 yr; [δ]CB *vs*. 5-10 yr; [%]CB *vs*. 10-16 yr; [¨]0-6 months *vs*. 6-12 months, [>]0-6 months *vs*. 1-2 yr; [z]0-6 months *vs*. 2-5 yr; [y]0-6 months *vs*. 5-10 yr; [x]0-6 months *vs*. 10-16 yr; [w]6-12 months *vs*. 1-2 yr; [v]6-12 months *vs*. 2-5 yr; [u]6-12 months *vs*. 5-10 yr; [t]6-12 months *vs*. 10-16 yr; [s]1-2 yr *vs*. 2-5 yr; [r]1-2 yr *vs*. 5-10 yr; [q]1-2 yr *vs*. 10-16 yr; [p]2-5 yr *vs*. 5-10 yr; [o]2-5 yr *vs*. 10-16 yr; [n]5-10 yr *vs*. 10-16 yr; [a]Per cent of leucocyte; [b]Per cent of peripheral lymphocytes; [c]Per cent of Th cells; [d]Per cent of Tc cells; [e]Per cent of B cells; [f]Per cent of T cells; [g]Per cent of NK cells; [h]Per cent of CD3+CD4-CD8-gd-T cells. CM, central memory; EM, effector memory; TEMRA, terminally differentiated effector memory re-expressing CD45RA; DNT, double negative T; Tc, T cytotoxic; Th, T helper; NK, natural killer; gd, gamma delta; TCRgd, T cell receptor gd; HLADR: human leukocyte antigen DR

Of particular interest was the observation regarding the CD4/CD8 ratio, which underwent a gradual decline with increasing age. Furthermore, the populations of naive T cells and recently thymic emigrants (RTE) along with Tc cells exhibit a progressive reduction with progressing age. In contrast, central memory and effector memory T cells increased in the later years of life. TEMRA (terminally differentiated effector memory RA+) on CD4 cells remained relatively low across various age groups; however, CD8 cells showed an increasing trend with age.

The relative frequency of T cell receptor gamma delta ($TCRgd^+$) cells increased till 16 yr of age, whereas double-negative $TCRgd^+$ T cells increased till 10 yr of age after which they declined. The $TCRgd^-$ T-cells showed an overall decreasing pattern and double-negative $TCRgd^-$ T-cells remained stable, showing a relative frequency of 0.68-1.85 per cent. T cell activation status as assessed by HLA-DR expression showed an increasing pattern up to one year following a gradual declines. CD45RA and HLA-DR co-expression accessed for double-negative T (DNT) $TCRgd^-$ cells was found to be 3-7.6 per cent. DNT cells were relatively stable.

It was observed that absolute B-cell count and the percentage decrease with age. Naive B-cells showed the same pattern, while B memory and un-switched cells showed an increasing absolute count up to two years followed by a gradual decrease. IgM-only memory B cells remain very low. There is an increase in class-switched memory B cells with age. NK cell counts are relatively stable across the age groups.

Discussion

Advances in multicolour flow cytometry allow one to look at an array of immune parameters which are typically evaluated in different disease situations such as autoimmunity, immunodeficiency, malignancy and infectious diseases. For interpretation of results, although there are several studies reporting reference values for basic lymphocyte subsets in different populations globally[4-7,14,16-19], a lack of paediatric age reference values for the extended lymphocyte subset is evident. To the best of our knowledge, there are no studies from India that give reference values for rare subsets for paediatric age groups such as naïve and memory T/B cell, RTE, activated T, activated NK cells, NKT cells, DNT cells, TCRgd+ T cells or HLADR and CD45RA co-expression on DNT cells.

In certain diseases, especially IEI, the basic lymphocyte subset may be normal, but individual subpopulations may be abnormal, and evaluation of

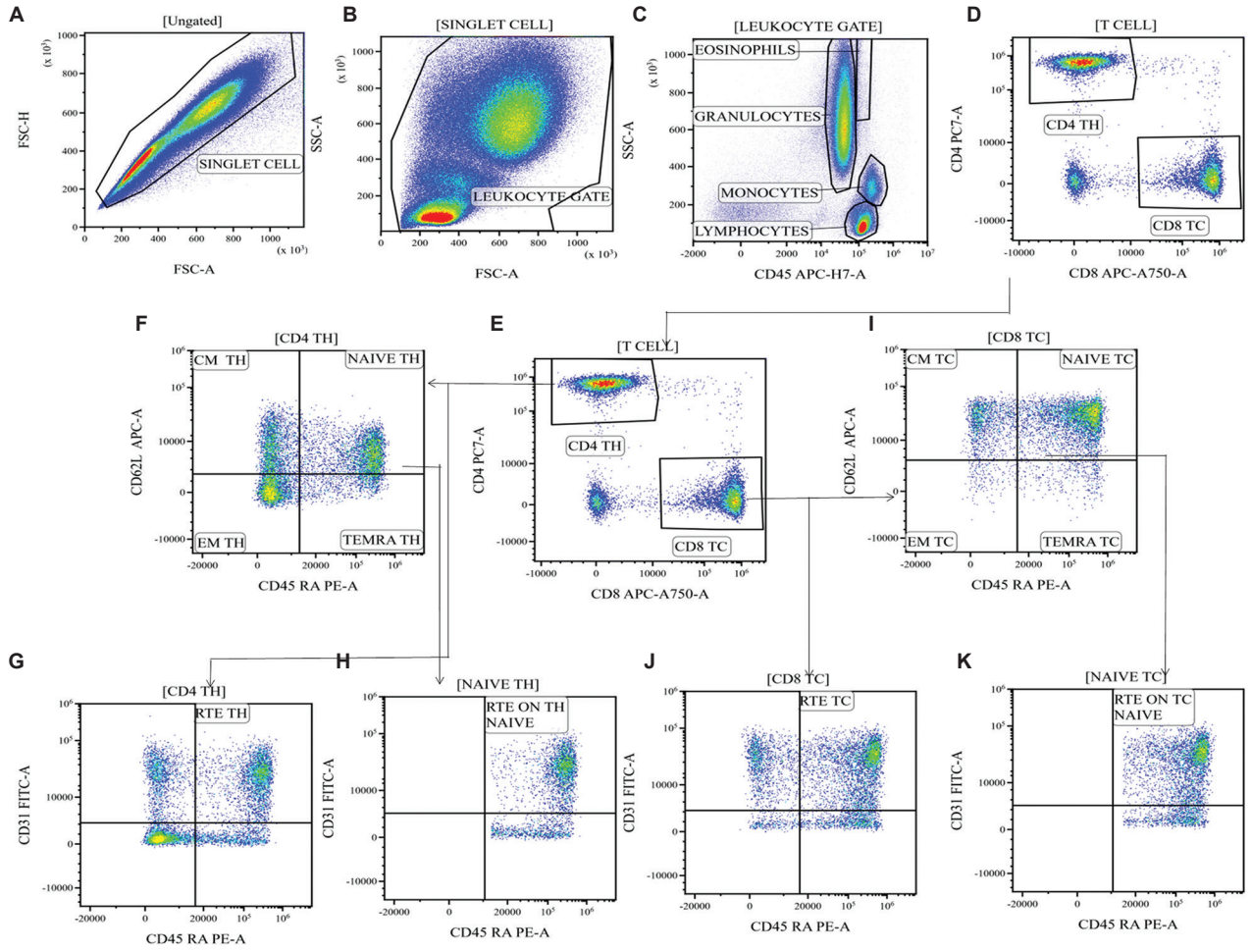

Fig. 2. Gating strategy for T cell and its subpopulation like Naïve, CM, EM, Terminally differentiated effector memory re-expressing CD45RA+ (TEMRA) and RTE cells. (**A**) FSC-H vs. FSC-A plot was used to remove doublets cell debris. (**B**) Singlet cells were further plotted against SSC-A vs FSC-A. (**C**) SSC-A vs. CD45 was used to gate lymphocytes, based on low forward /side scatter and bright CD45. (**D**) SSC-A vs. CD3 was used to identify T cells. (**E**) T cells were characterized as T helper cells/cytotoxic T cells based on the expression of CD4 and CD8. (**F and I**) Quadrant density plot representing naïve, central, effector memory and TEMRA sub population using CD62L and CD45RA on Th and Tc cells. (**G, J, H and K**) Recent thymic emigrants (RTE) were identified based on co-expression of CD31 and CD45RA and or CD62L on both Th and Tc cells. The names written above the plot (name) represent the parent gate. CM, Central memory; EM, effector memory; RTE, recent thymic emigrant

such subsets is important. In this study such reference ranges have been utilized in interpreting immunological parameters in patients with IEI, especially severe combined immunodeficiency (SCID), combined immunodeficiency (CID), predominant antibody deficiencies (PADs) and primary immune regulatory disorders[20-24].

To differentiate true lymphopenia from transient lymphopenia, *i.e.* if T-cells are low but naive T-cells counts are normal, it is suggestive of transient lymphopenia, whereas if both are on the lower side, it suggests SCID[25]. RTE plays a vital role in autoimmune diseases like rheumatoid factor-negative polyarticular juvenile idiopathic arthritis and in adults with systemic lupus erythematosus, type I diabetes and psoriasis[16]. CD8 RTE plays a major role in chronic viral diseases, its decrease has been associated with SCID and Ommens syndrome[26]. HLA-DR and CD45RA co-expression on DNT cells is reported to be elevated in autoimmune lymphoproliferative syndrome, signal transducer and activator of transcription 3-gain-of-function, and CTLA4 deficient patients[27].

Patients with PADs such as common variable immunodeficiency and CID-like hyper IgM can have normal B-cell count but reduced memory and class switch cells. DOCK-8 deficient patients show an

Fig. 3. Age-related ranges for absolute lymphocyte subset counts. Flow cytometric analysis of 148 healthy individuals amongst seven different age groups. All values of this reference dataset are represented as box plots with median, p10, p25, p75 and p90 percentile. For the data visualization package ggplot2 for the statistical language, R was used. Cb, cord blood; yr, years, m, month

increase in naïve and a decrease in memory B cells[28]. IEIs like PI3KCD-activated p110 δ syndrome can have B and T cell abnormalities such as low pre-GC memory B cells, class-switched memory B cells and skewing of CD4+ with CD8+ T cells towards terminally differentiated effector cells[1]. CD27 deficiency can be identified based on absent CD27 expression on naive T cells with no memory B cells.

We thus report pilot data for 34 immune parameters, both absolute as well as relative counts (Figs. 3 and 4). This preliminary study can be used as a pilot for future large scale multicentric study to determine the reference values that can help in making the diagnosis of several infectious and immunological diseases like IEI (Supplemantary Fig. 2) as well as monitoring the treatment outcome.

The lymphocyte subset reference ranges were compared among the American, Asian, European, African and Indian populations for available subsets[1,6,7,14,16,18,29] (Supplementary Table III). The majority of T, B and NK cell subsets showed no significant difference across the different populations (Supplementary Table III.). T helper cells were higher than in the Cameroonian population, and T cytotoxic cells were higher than in the African and American populations, as reported previously[7,14].

Due to the relative lymphocytosis in healthy infants, the corresponding Th and Tc cell counts were elevated for a year and then declined with age. Naïve T-cells can be identified using a combination of various markers such as CCR7, CD62L, CD27, CD28 and CD45RA[30]. We used a combination of CD62L and CD27 with CD45RA. Using unpaired t test, we found no significant difference between naïve helper and naïve cytotoxic populations gated on CD27 and CD62L, but memory and effector

Fig. 4. Age-related ranges for the percentage of lymphocyte subset counts. Flow cytometric analysis of 148 healthy individuals amongst seven different age groups. All values of this reference dataset are represented as box plots with median, p10, p25, p75, and p90 percentile. For the data visualization package ggplot2 for the statistical language, R was used. Cb, cord blood; yr, years; m, month.

group sub-populations did show a difference (Supplementary Table IV). Thus, naïve T cells can be identified using any of the markers along with CD45RA. Our data are consistent with known effects of development, including lower naïve Th and Tc cells and accumulating T cell subsets with a memory phenotype[3,16]. It reflects a gradual decline in the naïve T cells due to ageing and thymic involution and also the increased immunological memory due to exposure to environmental antigens [31]. In the absence of newborn screening for SCID in India, flow cytometric assessment of naïve T cells in suspected cases provides a mechanism for prompt diagnosis while awaiting molecular confirmation.

TCRgd+ T cell relative percentages reportedly increase with age, as seen in other populations. The absolute counts have been shown to increase up to 10 years of age followed by a decline, as seen in the Chinese population. The median cell count was found to be higher compared to the European population but was similar to that of the Chinese, suggesting an increased exposure to pathogens in the Asian population compared to Europeans[16,17].

Naive B cells have been shown to predominate in the peripheral B cell pool during infancy, and the fraction of class-switched and non-switched memory B cells increases gradually with age. B memory cell percentage rises until 10 yr of age followed by a marginal decrease similar to that of the European population[32,33]. The absolute count reportedly increases for two years after which there is a decline[32].

NK cell subsets were relatively stable overall. Previous studies have shown that diseases like XLP, SAP deficiency, have low NKT cell[28], whereas activated HLA-DR is increased in severe COVID -19 patients[34]. In our study we present the range for NKT

cells and activated HLA-DR expression ranges for healthy children which can be used in such diseases.

The present study has a few limitations; the most important being the low sample size in the younger age group and lack of consideration of region-wise variability as the study was single centric. We relied mostly on pre-surgical samples of benign procedures for the younger age group because it was difficult to get samples from these groups due to hesitation from parents to consent. A recent study from India and several international publications have shown no significant difference between males and females; thus, we did not compare our data separately[5,14,16,17]. Furthermore, we could not assess few T cells subsets such as Tregs, T follicular helper and Th1, Th2, Th17 and B cell subsets such as plasmablasts and transitional B cells.

Overall, this study provides preliminary data that justify the need for future large-scale multi-centric studies to generate a reference range for interpreting extended immunophenotyping profiles in the paediatric age group, making it possible for clinicians to assess the immunological status in IEI, infectious and autoimmune diseases.

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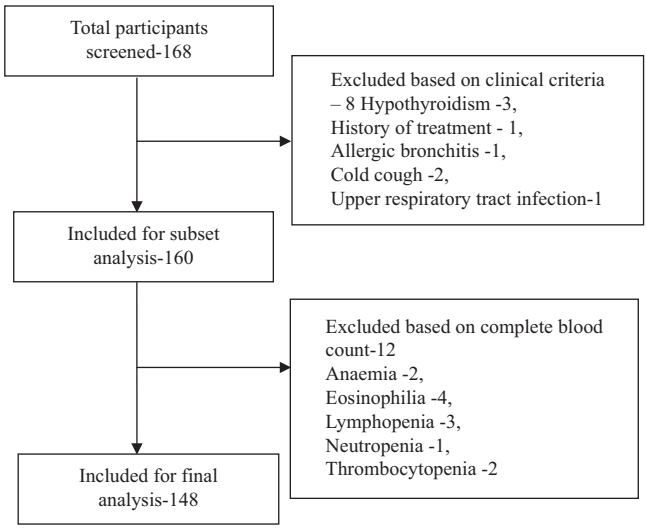
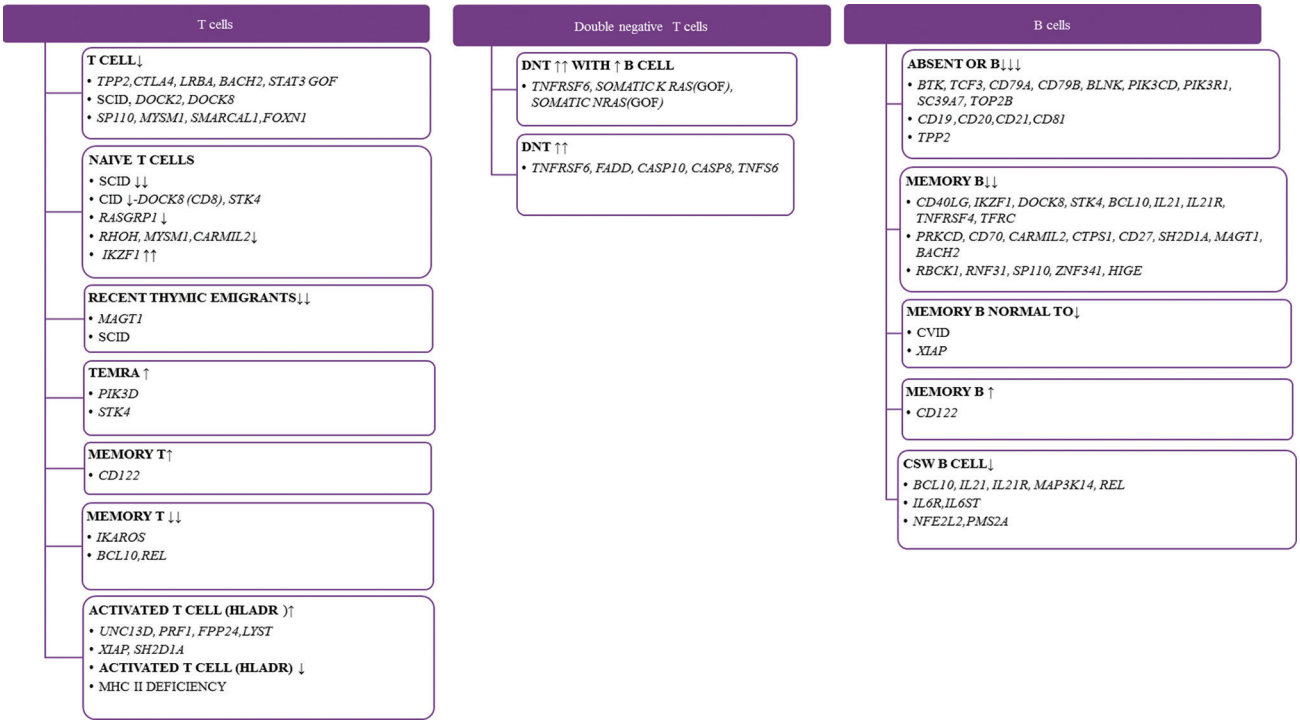


Supplementary Fig. 1. Consort diagram of inclusion of participants.



Supplementary Fig. 2. Application of age-matched ranges in diagnosing clinically suspicious IEI. IEI, inborn errors of immunity

Supplementary Table I. Cell surface markers used for analyzing peripheral blood lymphocyte subsets

Subset	Anchor marker	CD subset measured
T cell	CD45	CD3+
NK cell	CD45	CD3−CD16+CD56+
B cell	CD45	CD3−CD19+
Th cell	CD45	CD3+CD4+
Tc cell	CD45	CD3+CD8+
Th Naïve cell (CD27)	CD4	CD27+CD45RA+
Th RTE	CD4	CD31+CD45RA+
CM Th cell (CD27)	CD4	CD27+CD45RA−
EM Th cell (CD27)	CD4	CD27−CD45RA−
TEMRA Th cell (CD27)	CD4	CD27−CD45RA+
Tc Naïve cell (CD27)	CD8	CD27+CD45RA+
CM Tc cell (CD27)	CD8	CD27+CD45RA−
EM Tc cell (CD27)	CD8	CD27−CD45RA−
TEMRA Tc cell (CD27)	CD8	CD27−CD45RA+
Tc RTE	CD8	CD31+CD45RA+
RTE on Th Naïve	CD4	CD31+CD45RA+CD62L+
RTE on Tc Naïve	CD8	CD31+CD45RA+CD62L+
Total memory B cell	CD19	CD27+
Class switch memory B cell	CD19	CD27+IgM−IgD−
Unswitched memory B cell	CD19	CD27+IgM+IgD+
IgM only memory B cell	CD19	CD27+IgM+IgD−
Naïve B cells	CD19	CD27−IgD+
Pre-GC B cells	CD19	CD27−IgD+IgM+
Double negative B cell	CD19	CD27−IGD−
DNT on Lymphs	CD45	CD3+CD4−CD8−
DPT on Lymphs	CD45	CD3+CD4+CD8+
DNT ab+T	CD3	CD3+CD4−CD8−TCRgd−/TCRab+
DNT gd+T	CD3	CD3+CD4−CD8−TCRgd+
gd- T/ab+T	CD3	TCRgd−
gd+T	CD3	TCRgd+
NKT	CD45	CD3+CD16+CD56+
HLADR+Th cell	CD3	CD4+HLADR+
HLADR+Tc cell	CD3	CD8+HLADR+
HLADR+T cell	CD3	HLADR+
HLADR+NK cell	CD16/CD56	HLADR+
HLADR+45RA OF DNTgd-T	CD3	CD3+CD4−CD8−TCRgd−HLADR+CD45RA+
Th Naïve cell (CD62L)	CD4	CD62L+CD45RA+
CM Th cell (CD62L)	CD4	CD62L+CD45RA−
EM Th cell (CD62L)	CD4	CD62L−CD45RA−
TEMRA Th cell (CD62L)	CD4	CD62L−CD45RA+
Tc Naïve cell (CD62L)	CD8	CD62L+CD45RA+

Contd...

Subset	Anchor marker	CD subset measured
CM Tc cell (CD62L)	CD8	CD62L+CD45RA−
EM Tc cell (CD62L)	CD8	CD62L−CD45RA−
TEMRA Tc cell (CD62L)	CD8	CD62L−CD45RA+
gd+T of Lymphs	CD45	CD3+TCRgd+

TEMRA, terminally differentiated effector memory re-expressing CD45RA; CM, central memory; EM, effector memory; DNT, double negative T; NK, natural killer; gd, gamma delta; Tc, T cytotoxic; Th, T helper; HLADR, human leukocyte antigen DR

Supplementary Table II. Monoclonal antibody used for extended immunophenotyping

Cell type	Antibody	Fluorochrome	Clone	Maker
General	CD45	APCH7	J3-119	BC
lymphocyte	CD16+CD56	PE	3G8+N901	BD+BC
population	CD19	PC7	2D1	BD
	CD3	APC	UCHT1	BC
T cell	CD4	PERCP Cy5.5	SK-3	BD
subpopulation	CD8	FITC	SK-1	BC
	CD27	ECD	IA4CD27	BD
	TCRgd	PC7	IMMU-510	BC
	HLADR	PACIFIC BLUE	IMMU-357	BC
	CD45RA	BV510	H100	BD
B cell	IgD	FITC	IA6-2	BIOLEGEND
subpopulation	CD27	ECD	IA4CD27	BC
	IgM	PERCP Cy5.5	MHM-88	BIOLEGEND
RTE	CD31	FITC	5.6E.	BC
	CD45RA	PE	ALB11	BC
	CD3	ECD	UCHT1	BC
	CD4	PC7	SFCI12T4D11	BC
	CD62L	APC	DREG-56	BD
	CD8	APC750	B9.11	BC

RTE, recent thymic emigrants; HLADR, human leukocyte antigen DR

Supplementary Table III. Comparison of reference values of absolute counts of lymphocyte subsets in children amongst different countries

Subset/ country	CB	0-6 m	6-12 m	1-2 yr	2-5 yr	5-10 yr	10-16 yr
Lymphocyte	3627	6013	6029	5210	4226	3108	3087
NIIH (present study)	2809-5917	3744-8637	3537-8294	3182-7410	3048-6752	2258-4781	2038-4113
Euroflow[1]	2040-5688	2443-9955	4821-8531	2356-13275	1620-6856	1827-4564	1238-4792
USA	NA	5400 (3400-7600) 6300 (3900-9000)	5900 (3400-9000)	5500 (3600-8900)	3600 (2300-5400)	2700 (1900-3700)	2200 (1400-3300)
Italy[6]	NA	5740 (4054-7048)	5690 (3320-7006)	4685 (3873-6141)	3800 (2340-5028)	2500 (1662-3448)	2285 (1340-3173)
China male[17]	NA	5310 (3680-7340)	5890 (3730-8760)	4440 (2790-6350)	2940 (2280-3820)	2530 (2020-3610)	2440 (1780-3440)
China female[17]	NA	5120 (4020-6450)	5710 (3780-8110)	4390 (2980-5950)	3040 (2370-4290)	2650 (2020-3500)	2490 (1760-3000)
Netherland[16]	5400 (3100-9400)	6500 (3400-12200)	6300 (3200-12300)	4100 (1400-12100)	2700 (1400-5500)	2400 (1200-4700)	2400 (1400-4200)
T cell	2423	4321	3952	3642	2744	2300	2291
NIIH	1889-4764	2617-5380	2252-5788	2105-5460	2087-4899	1639-3388	1390-3008
India[14,29]	2402.38 (1725-3406)	3421 (952-8586)	4630 (1623-8159)	3801 (1480-6475)	3110 (1191-6692)	2347 (1191-4497)	1960 (1035-4493)
Euroflow[1]	1186-4113	1680-7754	3764-6289	1900-9345	852-5333	1352-3275	930-3477
USA[7]	NA	3930 (2500-5600)	3930 (1900-5900)	3550 (2100-6200)	2390 (1400-3700)	1820 (1200-2600)	1480 (1000-2200)
Italy[6]	NA	4040 (3180-5401)	3833 (2284-4776)	3133 (2542-4933)	2580 (1578-3707)	1793 (1239-2611)	1629 (954-2332
India[19]	2859 (3100-5200)	3631 (1767-5495)	2994 (1278-4710)	2506 (1222-3790)	2590 (1368-3812)	NA	NA
Cameroon[18]	2249 (430-2977)	3085 (2352-4776) 3120 (1123-4378)	3522 (2039-5024)	2639 (794-3307)	2299 (1159-3242)	NA	NA
China[17]	NA	3391 (1885-4954)	3625 (1571-7165)	2816 (1355-4921)	2028 (1254-3216)	1758 (1093-3013)	1649 (999-2607)
Netherland[16]	3100 (1400-6800)	4500 (2200-9200)	4400 (2400-8300)	2500 (700-8800)	1900 (850-4300)	1800 (770-4000)	1600 (850-3200)
Th cell	1637	2802	2274	2090	1463	1109	1168
NIIH	1060-3558	1336-3327	1207-4013	1154-3460	1001-2535	805-1855	659-1547
India[14,29]	1808 (1260-2440)	2156 (659-6132)	2852 (913-5680)	2271 (817-4893)	1821 (794-4323)	1266 (618-2555)	1080 (582-2045)
Euroflow[1]	952-3097	1273-5633	2093-4769	617-5959	516-3448	776-1815	576-1891
USA[7]	NA	2610 (1600-4000) 2850 (1800-4000)	2670 (1400-4300)	2160 (1300-3400)	1380 (700-2200)	980 (650-1500)	840 (530-1300)

Contd...

Subset/ country	CB	0-6 m	6-12 m	1-2 yr	2-5 yr	5-10 yr	10-16 yr
Italy[6]	NA	3079 (2330-3617)	2492 (1523-3472)	1866 (1573-2949)	1448 (870-2144)	1030 (646-1515)	887 (610-1446)
Cameroon[18]	1552 (330-1995)	2001 (1642-3472) 2133 (759-3205)	2252 (1311-3273)	1667 (596-1949)	1289 (674-1721)	NA	NA
India[19]	1707 (2209-3205)	2932 (1516-4348)	2427 (1056-3799)	2029 (1113-2946)	1977 (839-3115)	NA	NA
China[17]	NA	2287 (1217-3422)	2318 (850-4658)	1472 (717-2798)	1001 (546-1768)	839 (496-1479)	781 (391-1421)
Netherland[16]	2200 (1000-4800)	3300 (1600-6500)	3000 (1300-7100)	1600 (400-7200)	1100 (500-2700)	1000 (400-2500)	900 (400-2100)
Tc cell	707	1010	1315	1185	1057	856	841
NIIH	451-1346	618-1778	548-1930	781-1692	562-1809	508-1257	471-1242
India[14,29]	721 (558-1064)	970 (159-3717)	1407 (455-3393)	1319 (549-2844)	1084 (315-2258)	913 (422-1878)	767 (405-2615)
Euroflow[1]	213-1138	354-2006	720-1271	364-2498	188-1805	366-1171	261-1189
USA[7]	NA	980 (560-1700) 1050 (590-1600)	1040 (500-1700)	1040 (620-2000)	840 (490-1300)	680 (370-1100)	530 (330-920)
Italy[6]	NA	1048 (712-1361)	976 (524-1583)	884 (656-1432)	804 (472-1107	595 (365-945)	518 (282-749)
Cameroon[18]	554 (140-1188)	790 (570-1714) 726 (298-1413)	995 (563-1796)	823 (194-1165)	775 (308-1249)	NA	NA
India[19]	836 (312-1360)	1544 (970-2118)	1133 (541-2807)	1269 (523-2015)	1373 (749-1997)	NA	NA
China[17]	NA	963 (436-1846)	1212 (472-2874)	1047 (397-2080)	765 (455-1430)	709 (396-1325)	699 (366-1091)
Netherland[16]	800 (200-2700)	1000 (300-3400)	1200 (400-4100)	700 (200-2800)	600 (200-1800)	600 (200-1700)	600 (300-1300)
Th Naïve cell	1445	2619	1797	1552	1057	825	644
NIIH	1001-3329	993-3086	758-2992	816-2509	711-1844	416-1462	325-1208
Euroflow[1]	920-2897	1092-5337	1748-4201	360-5273	276-2902	424-1393	264-1484
USA[7]	NA	2250 (1200-3600) 2230 (1300-3600)	2100 (1100-3600)	1640 (950-2800)	960 (420-1500)	560 (310-1000)	390 (210-750)
China male[17]	NA	1839 (1170-2595)	1802 (764-2972)	918 (472-1760)	595 (321-972)	407 (294-683)	410 (230-627)
China female[17]	NA	1908 (1433-2546)	1967 (1042-3160)	929 (530-1837)	633 (339-1037)	489 (299-857)	442 (276-654)
Netherland[16]	1800 (900-3900)	3100 (1600-6000)	2700 (1100-6400)	1300 (200-7500)	800 (300-2300)	700 (200-2500)	600 (200-1700)
Th Naïve cell (CD62L)	1503	2177	1811	1578	957	632	564
NIIH	944-3371	875-2896	793-3181	791-2812	627-1570	388-1268	285-995
Cameroon[18] (CD62L)	1201 (693-1589)	1392 (798-2304) 1304 (453-2134)	1541 (763-2538)	708 (282-1559)	665 (395-1334)	NA	NA

Contd...

Subset/ country	CB	0-6 m	6-12 m	1-2 yr	2-5 yr	5-10 yr	10-16 yr
Tc Naïve cell	624	869	773	757	790	537	414
NIIH	418-1206	434-1135	410-1223	437-1185	456-1293	306-844	244-829
Euroflow[1]	200-1010	330-1841	564-1040	222-2178	126-1130	175-730	94-986
USA[7]	NA	730 (380-1300) 740 (450-1200)	700 (330-1200)	760 (400-1400)	540 (260-850)	410 (200-650)	300 (170-560)
China male[17]	NA	800 (503-1276)	909 (535-1677)	653 (356-1095)	462 (297-730)	380 (245-657)	375 (231-568)
China female[17]	NA	741 (484-1009)	726 (461-1235)	589 (295-971)	447 (293-768)	387 (232-665)	328 (210-560)
Netherland[16]	360 (23-1300)	690 (290-1650)	580 (140-2460)	310 (30-3100)	240 (53-1100)	240 (42-1300)	220 (78-640)
Tc Naïve cell (CD62L)	656	823	754	821	791	633.5	470
NIIH	419-1146	369-1169	374-1307	506-1243	405-1233	301-991	230-653
Cameroon[18] (CD62L)	457 (91-826)	545 (272-809) 404 (219-757)	524 (236-1042)	330 (120-555)	384 (156-641)	NA	NA
B cell	628	1912	1405	1124	815	440	556
NIIH	255-972	240-2636	715-2531	718-2007	510-1662	286-924	273-813
India[14,29]	518 (366-697)	1654 (351-5946)	1915 (523-3799)	1484 (246-4139)	1187 (362-2754)	653 (295-1650)	507 (115-1117)
Euroflow[1]	347-1053	470-4327	896-2316	353-2300	232-1637	157-725	173-1194
USA[7]	NA	1550 (430-3000)	1520 (610-2600)	1310 (720-2600)	750 (390-1400)	480 (270-860)	300 (110-570)
Cameroon[18]	552 (85-853)	1525 (513-3748) 1940 (696-3370)	1858 (662-2870)	1526 (487-2066)	965 (432-1691)	NA	NA
India[19]	1175.7 (61-2447.5)	1537.9 (860-2215.8)	746.3 (25-1809)	719.3 (88-1576.8)	776.9 (56-1697.3)	NA	NA
China[7]	NA	1185 (113-2317)	1254 (522-2175)	839 (359-2037)	443 (231-910)	347 (154-685)	312 (141-534)
Netherlands[16]	540 (140-2000)	1100 (520-2300)	940 (110-7700)	760 (160-3700)	490 (180-1300)	290 (100-800)	300 (120-740)
Italy[6]	NA	1032 (315-1383)	1123 (776-2238)	1152 (733-1338)	730 (434-1274)	403 (276-640)	321 (173-685)
Netherland[16]	NA	1623 (961-3679)	1717 (571-3680)	1157 (686-1732)	593 (278-1022)	338 (116-555)	284 (119-578)
NK cell	250	338	297	342	325	258	238
NIIH	69-1412	220-779	84-1237	144-565	130-636	90-679	110-437
India[14,29]	760 (450-1059)	489 (114-1624)	433 (105-1088)	368 (114-1201)	335 (131-1163)	362 (124-1005)	334 (78-774)
Euroflow[1]	200-1305	167-1359	237-1146	104-2436	138-1759	106-1348	109-1021
USA[7]	NA	420 (170-1100)	400 (160-950)	360 (180-920)	300 (130-720)	230 (100-480)	190 (70-480)
Italy[6]	NA	408 (208-1700)	381 (230-801)	296 (186-724)	299 (155-565)	262 (120-483)	230 (87-504)

Contd...

Subset/ country	CB	0-6 m	6-12 m	1-2 yr	2-5 yr	5-10 yr	10-16 yr
Cameroon[18]	496 (135-969)	546 (262-1030) 450 (140-1229)	504 (109-831)	397 (151-848)	333 (133-1042)	NA	NA
China[17]	NA	446 (168-1156)	505 (179-1522)	499 (166-1669)	418 (181-1000)	394 (119-1077)	424 (121-1064)
Netherlands[16]	120 (500-3100)	440 (97-1990)	500 (71-3500)	470 (55-4000)	180 (61-510)	200 (70-590)	330 (92-1200)
Activated Th 4/DR	7	45	53	35	27	31	39
NIIH	2-24	32-120	22-76	16-117	17-97	17-64	13-67
Cameroon[18]	21 (9-82)	75 (56-164) 74 (29-155)	77 (45-209)	70 (29-127)	65 (34-122)	NA	NA
USA[7]	NA	150 (60-280)	120 (50-260)	130 (70-280)	90 (50-180)	70 (40-120)	60 (30-100)
Activated Tc 8/DR	3	142	101	108	41.5	50	50
NIIH	1-8	13-295	14-263	21-209	14-195	20-124	9-139
Cameroon[18]	6 (2-25)	34 (8-287) 45 (13-324)	102 (30-342)	81 (21-216)	77 (39-307)	NA	NA
USA[7]	NA	80 (30-170)	90 (40-290)	180 (60-600)	140 (70-420)	90 (40-270)	70 (30-180)
TCRgd+T cell	68	191	246	206	241	189	189
NIIH	26-174	119-545	73-558	135-340	133-406	97-390	64-353
Euroflow[1]	18-121	25-435	128-335	86-537	44-784	66-416	56-332
Netherlands[16]	87 (30-250)	170 (56-510)	210 (70-630)	160 (41-640)	160 (27-960)	160 (27-960)	150 (39-540)
China male[17]	97 (51-240)	141 (92-279)	238 (128-436)	267 (114-539)	233 (124-410)	210 (124-388)	198 (81-343)
China female[17]	139 (71-356)	187 (94-301)	205 (143-409)	283 (128-520)	243 (134-428)	234 (121-462)	176 (85-358)
DNT gd-/ ab+T	26	51	59	88	85	45	40
NIIH	14-56	17-149	30-105	42-137	48-125	30-90	26-65
Euroflow[1]	0-37	9-66	15-73	20-141	3-104	13-80	8-53
Netherlands[16]	28 (10-79)	22 (5.5-140)	33 (11-100)	48 (13-170)	47 (16-140)	32 (1-100)	27 (9-78)
China male[17]	20 (11-26)	18 (11-45)	29 (16-58)	27 (9-57)	23 (4-55)	26 (13-48)	23 (12-37)
China female[17]	17 (6-35)	23 (13-38)	37 (19-72)	31 (16-58)	26 (4-49)	23 (12-41)	22 (13-44)

Contd...

Subset/ country	CB	0-6 m	6-12 m	1-2 yr	2-5 yr	5-10 yr	10-16 yr
Th RTE	1297	1905	1625	1375	806	570	459
NIIH	842-2911	766-2563	593-2977	701-2388	522-1456	342-1126	227-860
Spain[3]	NA	1440 (600-2700)	1440 (600-2700)	1440 (600-2700)	800 (600-1600)	590 (300-1000)	520 (300-700)
Netherlands[16]	1700 (710-4200)	2700 (1400-5200)	2200 (800-6200)	1100 (170-7400)	710 (190-2600)	590 (200-1700)	480 (150-1500)

Data of absolute numbers represented as median and (10^{th} to 90^{th}) or (5-95^{th}) percentiles. Data sources: NIIH-this study, Euroflow[1]; Netherlands[16]; China[17]; Spain[3]; Italy[6]; Cameroon[18]; India Thakar *et al.*[14]; India Prabhu *et al.*[29]; India Narula *et al.*[19]; Thakur *et al*: from 0 to 16 yr; Prabhu *et al*: CB. NA, not available; NIIH, National Institute of Immuno Haematology; CB, cord blood, RTE, recent thymic emigrants; DNT, double negative T; gd, gamma delta; TCRgd, T cell receptor gd; Tc, T cytotoxic; Th, T helper; HLADR, human leukocyte antigen DR

Supplementary Table IV. Comparison between T helper and T cytotoxic subsets using CD62L versus CD27 as gating marker as assessed by unpaired *t*-test

t test	CD4				CD8			
	Naive	CM	EM	TEMRA	Naive	CM	EM	TEMRA
CB	0.788	0.1222	<0.0001	<0.0001	0.9941	0.0054	0.0064	0.0084
0-6 months	0.4916	0.6512	0.052	0.2518	0.8375	0.7936	0.625	0.9072
6-12 months	0.6982	0.1677	0.0009	0.0387	0.9156	0.06	0.2484	0.7008
1-2 yr	0.8901	0.1166	<0.0001	0.0619	0.814	0.0517	0.0027	0.7298
2-5 yr	0.3292	0.0009	<0.0001	0.0007	0.8584	0.0978	0.0088	0.9822
5-10 yr	0.1465	0.0051	<0.0001	0.0236	0.3236	0.0854	0.0005	0.0037
10-16 yr	0.2209	0.0783	<0.0001	0.6732	0.9839	0.0848	0.0337	0.4895

The boxes highlighted in yellow represents non-significant differences. CM, central memory; EM, effector memory; TEMRA, terminally differentiated effector memory re-expressing CD45RA; CB, cord blood