RESEARCH PAPER

Reference Ranges of Different Lymphocyte Subsets in Indian Children: A Multi-Centric Study

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Objective: To determine the reference ranges of various lymphocyte subsets in healthy Indian children.

Design: Descriptive cross-sectional study.

Setting: Four centers in India representing four geographical regions.

Participants: 1104 children from neonatal age to 18 years of age.
Measurement: One time measurement of absolute count and percentages of different lymphocyte subsets i.e. T lymphocytes (CD3+T, CD4+T, CD8+T cells), B lymphocytes (CD19+B cells) and Natural Killer lymphocytes (CD15/16+NK cells) in whole blood using multicolor flow cytometry.

Results: The absolute cell counts of various lymphocytes were found to increase from newborn to 10 months of age, followed by gradual decline until 18 years; however, the proportion of immune cells remained largely similar. Gender did not have a significant impact on the reference ranges, whereas counts were found to vary as per the geographical locations.

Conclusions: These reference ranges will be useful to monitor and predict the immune status in pediatric population. The variation in region wise ranges could be confirmed by testing more number of samples in the specific age groups.

Keywords: *Flow cytometry, NK cell, CD4+T cells, B cells.*

Cellular differentiation pathways in children are distinctly different form adults [1]. Additionally, the cellular immune component of the blood is known to be dynamic and showing variable frequencies of different immune subsets at different ages especially in pediatric population [7]. In India, although the lymphocytic reference ranges are available for healthy adults [2], there is not much data available on the reference range of lymphocytic subsets among pediatric population [3]. Since the ethnicity, age and environmental factors are known to influence the lymphocytic reference ranges [4-6], the available reference ranges from other countries cannot be used for the Indian population. Considering the variations in ethnicity across various geographic regions in India, it is important to generate the reference values in different pediatric age groups from across the country

In this study, we determined the age group specific values for major lymphocyte subsets among healthy pediatric population aged from newborn to 18 years across different geographical regions in India.

METHODS

This cross-sectional study was aimed at determining the reference values of lymphocyte subsets in healthy Indian children aged 0 through 18 years from four geographically diverse sites i.e., Bengaluru, Chandigarh, Mumbai and Kolkata, in order to obtain the data representative of the entire country. The different age groups included in the study were: Group I- Newborn; Group II - 6 weeks of age (before first DPT vaccination); Group III - 9 to 10 months of age (before measles vaccination); Group IV - 15 to 18 months of age or before first booster of DPT; Group V - 19 months to 5 of age, Group VI - > 5 to 12 years of age; and, Group VII - 12 to 18 years of age. The immunization visits coincided with the blood collection visits.

For group I, cord blood was used as a sample. For this group, babies with full term normal vaginal delivery or elective caesarean with or without mild anemia in pregnancy were included. Emergency caesarean cases, complicated deliveries with chronic illness or with infections, conditions like diabetes, toxemia, bleeding,

fever in mother, prolonged rupture of membrane, and HIV positive pregnancy were excluded from this study.

For other groups (Group II to VII), the inclusion criteria for healthy children in the different age group were: no history of cold and cough (for the last one month), blood transfusion (preceding 3 months), surgery (preceding 6 months) and recent diarrhea (4-6 weeks), born to HIV negative mother, and grade 1 malnutrition/ normal weight (weight-for-age >70th centile IAP chart). Children with moderate to severe anemia, acute or chronic infectious diseases (gastrointestinal diseases within the last 6 months) or any clinically significant disease or findings in the medical history that might compromise the study measures (e.g., diabetes mellitus, asthma, rheumatoid arthritis, cystitis fibrosis) were excluded from the study.

Children were enrolled after obtaining written consent from their parents, and assent, if required. The study was approved by the ethics committees of respective study sites. From each site, 50 children were enrolled in each group and in each age group an attempt was made to enroll boys and girls in a 1:1 ratio. The enrollment for groups I, II, III and IV was done in the hospitals (well-baby clinics of the hospitals) and for groups V, VI and VII, school-going healthy children were enrolled after obtaining appropriate permission from the school. In one day, not more than 5-7 eligible newborns/ infants/ children were enrolled in each group (Groups I to IV). For the groups V, VI and VII, the schools were contacted and the eligible participants were enrolled sequentially. The data on age, sex, place of origin, height, weight, nutritional status and vaccination was collected wherever possible.

Two to five milliliter of whole blood specimens were collected from the children in K3 EDTA evacuated tubes and were processed for immune-phenotyping the same day. To avoid diurnal variation, the samples were uniformly collected in the forenoon at all the study sites.

Immunophenotyping: The enumeration of different lymphocyte subsets were done by multicolor flow cytometry. The single platform technology was used to obtain both the absolute counts and percentages. All the centers used the same reagents, equipments and the standard operating procedure to obtain comparable data. Briefly, in the two separate Trucount tubes, 50 µL of whole blood and 20 µL of liquid antibody reagents (CD3 FITC, CD8 PE, CD45 PerCP, CD4 APC) or (CD3 FITC, CD16+56 PE, CD45 PerCP, CD19 APC) was added. All reagents were from the Becton Dickinson. The tubes were incubated at room temperature in dark for 15 minutes. Lysis of the red blood cells was carried out using 450 µl of 1:10 diluted FACS lysing solution). A total of 100000 cells were acquired in a FACSCalibur (BD Bio-sciences) and analyzed using Multiset software (BD Biosciences). The absolute count and percentage of the lymphocyte subsets in the gate $CD45_{high}/SSC_{low}$ i.e., the count or the percentage from the total lymphocyte population was calculated by the Multiset software. B lymphocytes were identified as CD19+, T lymphocytes as CD3+ and further differentiated as CD4+ and CD8+ T cells and NK cells were identified as CD3-CD16/CD56+cells

The optical alignment of the equipment and fluorescence compensation settings were ensured daily by running the calibration beads (CaliBRITE 3) and the compensation was done using the FACSComp software. Additionally, each center successfully participated in National external proficiency testing programmed for CD4 count estimation.

Data analyses: To determine normal ranges of lymphocyte parameters, 2.5 and 97.5 percentile values were calculated, which covers 95% of the population [8]. The age, gender and region specific ranges were also reported. Any differences in lymphocyte subsets within the geographic regions were assessed using Kruskal-Wallis Chi square test. The region-wise value for each parameter in each age group were compared with the overall reference range of the respective parameter using Mann-Whitney U test. *P* value of <0.05 was considered as significant. Analyses were done using IBM SPSS 24.0.

RESULTS

A total of 1674 children were enrolled across the four regions. Of these, data collected from 1104 subjects was considered for analysis; 570 samples were excluded due to various reasons like quality of samples, fail to fit in hemoglobin, BMI or weight criterion etc. Region- and age-wise numbers of subjects in each group are shown in **Table I**. The representation of the samples in groups II (6.1%) and III (11.3%) were lower as compared to the other groups. The median (range) weight and hemoglobin of the newborns was 3 (2.5-4.5) kg and 16.1(13-22.1) g/ dL, respectively. The hemoglobin decreased to 11.8 (11-15.1) g/dL in group II, but remained similar in older age groups. The median (range) body mass index was 16.6 (13.9-19.6) in group II, which remained similar in older age groups.

The median and 2.5th and 97.5th percentiles of absolute counts and frequencies (% populations) of various lymphocyte subsets; CD3+, CD4+ and CD8+ T cells, B cells and NK cells in seven different age groups are presented in **Table II** and **Fig. 1**.

Table I Regional Distribution of the Study Participants (*N*=1104)

	East *n=317*[a]	*North* *n=281*[b]	*South* *n=304*[c]	*West* *n=202*[d]
Newborn, *n*=194	50 (25.8)	51 (26.3)	49 (25.3)	44 (22.7)
6-32 wk, *n*=67	23 (34.3)	39 (58.2)	0	5 (7.5)
9-10 mo, *n*=125	49 (39.2)	24 (19.2)	49 (39.2)	3 (2.4)
15-18 mo, *n*=132	46 (34.8)	28 (21.2)	47 (35.6)	11 (8.3)
19 mo-5 y, *n*=197	54 (27.4)	44 (22.3)	50 (25.4)	49 (24.9)
5-12 y, *n*=210	44 (21)	55 (26.2)	59 (28.1)	52 (24.8)
12-18 y, *n*=17[a]	51 (28.5)	40 (22.3)	50 (27.9)	38 (21.2)

No. of boys in each region: [a]*176,* [b]*138,* [c]*145 and* [d]*101.*

The absolute counts of CD3+, CD8+, CD4+ T cells and CD19+B cells increased during the first few months till 9-10 months and decreased gradually from 15-18 months onwards till 12-18 years while NK cells showed a gradual decline in the absolute count post 6 weeks of birth till 15-18 months of age and then plateaued (**Fig. 1a**). The percentage values of CD3+, CD8+, CD4+ T cells (**Fig. 1b**) along with the ratio of CD4 and CD8 largely remained unchanged across different pediatric age groups. The percentage of CD19+ B cells however increased from 6 weeks to 5 years and later decrease in age group of 5-12 years and further in 12-18 years of age. The percentage of NK cells started to decline from 6 weeks onwards till 5 years of age and later increased and reached to the levels present in new born babies (**Fig. 1b**).

The female: male ratio was similar across different age groups (range: 1: 0.9 to 1: 1.19). The age-specific overall ranges (**Table II**) were compared with the gender-wise ranges in each age group for each parameter. We found no significant difference in the reference values observed in male and female children for any parameter in any age group.

Similarly we also compared the region-wise reference values with the overall reference ranges within every age group for each parameter. The significant difference was observed in case of group I (newborn), group IV (15th 18 months of age), group V (19 months to 5 years), group VI (5 years to 12 years) and group VII (12 years to 18 Years) for a few parameters. In newborn group, the values were

Table II Median and Reference Range of Different Immune Cells in Indian Healthy Children of Different Age Groups (*N*=1104)

	Newborn *n*=194	*6 -32 wk* *n*=67	*9 -10 mo* *n*=125	*15-18 mo* *n*=132	*19 mo-5 y* *n*=197	*5-12 y* *n*=210	*12-18 y* *n*=179
CD3+cells							
Absolute counts	2731 (979-5024)	3421 (952-8586)	4630 (1623-8159)	3801 (1480-6475)	3110 (1191- 6692)	2347 (1191-4497)	1960 (1035-4493)
Percentage	65 (42-85)	63 (38-78)	63 (45-76)	64 (37-77)	63 (51-75)	67 (51-77)	66 (54-89)
CD4+cells							
Absolute counts	1827 (601-3243)	2156 (659-6132)	2852 (913-5680)	2271 (817-4893)	1821 (794-4323)	1266 (618-2555)	1080 (582-2045)
Percentage	43(23-58)	44 (15-60)	39 (24-58)	38 (24-53)	39 (26-50)	37 (26-50)	36 (26-520)
CD8+cells							
Absolute counts	881 (337-1889)	970 (159-3717)	1407 (455-3393)	1319 (549-2844)	1084 (315-2258)	913 (422-1878)	767 (405-2615)
Percentage	20 (11-40)	18 (7-42)	20 (10-37)	22 (10-40)	23 (13-32)	26 (17-38)	26 (17-52)
CD4/ CD8 ratio	2.0 (0.8-4.6)	2.4 (0.5-7.0)	2.0 (0.8-5.5)	1.7 (0.6-3.7)	1.8 (0.9-3.3)	1.4 (0.8-2.4)	1.4 (0.6-2.4)
CD19+cells							
Absolute counts	760 (70-2532)	1654 (351-5946)	1915 (523-3799)	1484 (246-4139)	1187 (362-2754)	653 (295-1650)	507 (115-1117)
Percentage	18 (4-43)	27 (11-44)	27 (13-42)	26 (8-42)	25 (16-37)	19 (11-33)	18 (4-29)
CD16+/56+ cells							
Absolute counts	499 (125)	489 (114-1624)	433 (105-1088)	368 (114-1201)	335 (131-1163)	362 (124-1005)	334 (78-774)
Percentage	12 (4-36)	8 (2-18)	6 (2-16)	6 (3-15)	7 (3-17)	10 (4-26)	11 (3-24)

All values in median (RR); RR-Reference ranges (2.5- 97.5 percentile).

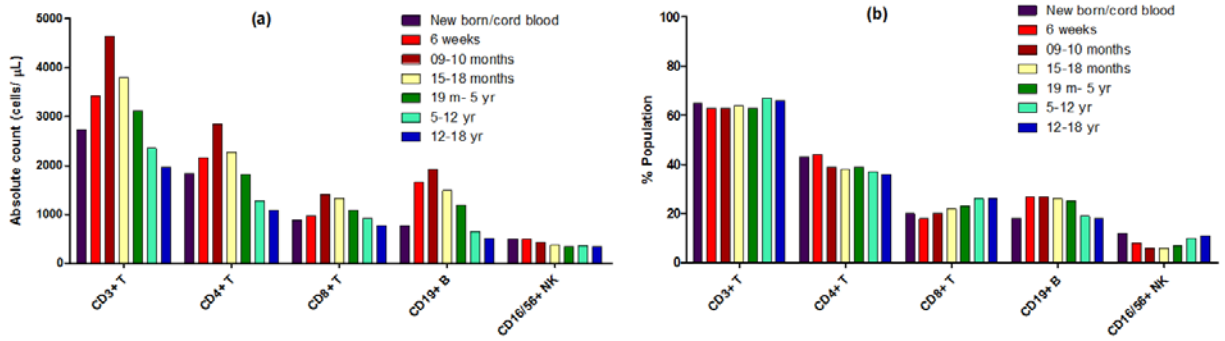

Fig. 1 The median values of each lymphocyte subsets in study groups. (a) Median values of absoplute counts, and (b) proportion of CD3+ CD4+, CD8+, CD19+ and CD16-56+ NK cells in all seven study groups.

significantly different in all lymphocyte subsets where as for other groups the values were different for eg in CD3, CD8 and CD19 percentages and absolute CD4 counts. The NK cell values were generally similar showing difference only in group I in case of North and South regions and in group VII in East and South regions (**Suppl. Table I**). Due to insufficient number of study participants from West region in groups II, III and IV, and from South region in groups II, the comparisons could not be made.

DISCUSSION

In this multi-centric study, we determined the reference ranges for different lymphocyte subsets in Indian pediatric population. This study represents the largest dataset for the relative frequencies of major lymphocyte subsets in healthy Indian children at various age groups from birth till 18 years of age. Unlike CD4+ and CD8+ T cells reference ranges, limited information is available on other lymphocyte subsets like CD3+T cells, CD19+ B cells and CD16/56+ NK cells which have important immune functions.

Our observations confirmed the previous findings that the lymphocyte compartments of normal healthy children differ considerably in various age groups [8-11]. The absolute T cell and B cell count increased during the first few months till 9-10 months and decreased gradually from 15-18 months onwards till 12-18 years. Whereas the relative percentages of T cells i.e. CD3+, CD8+ and CD4+ cells remained more or less similar in all age groups. Similar findings have been reported in African and Caucasian populations previously [10], and from children from southern India [3].

We found that the reference ranges in our cohort differ from pediatric population from other regions like Europe [6,8,12], Africa [13,14], and North America [11,15]. Among the newborns, the absolute cell counts for CD3+, CD4+, CD8+, CD19+ B cells were lower than the cohort from Italy [8] but higher than the African cohort from Cameroon [14]. These differences could be due to the differences in the total lymphocyte percentages and absolute counts, which were not measured in both the studies. Similarly the percentages of CD3+, CD4+ cells of the newborns were lower than Italian children [8] but higher than Cameroon [14], while the percentages of CD8+ T and CD19+ B cells were higher in our newborn group than the newborns from Italy and Cameroon. The number of samples tested from the newborn group might be the reason for such differences. The Italian study used 16 samples from the 0-3 month group whereas the Cameroon study used 38 cord blood samples. In other age groups of children in our study, the absolute CD4 counts were higher than seen in children from Europe, Africa and USA; however in children from 6 years to 18 years, it was comparable to children from Uganda [16]. Except in newborns, children in all age groups had a higher CD8 cell counts when compared with the children from Europe (Italy), Africa (Tanzania, Uganda, Cameroon) and USA [8,11,14,16]. The CD19+ B cell and NK cell counts were higher than the counts observed from Italian population [8] and largely comparable with the pediatric population from Cameroon [14]. Rathore, et al. [17] compared the different immune cells subsets in newborns from United States and India and found that Indian newborns had higher NK and CD4+ T cells, while lower subsets of total T cells, than the American cohort. In comparison with these values, the data from the present study showed lower CD4 counts whereas the CD8+ T cells, B cells, NK cell counts were in similar ranges. Similar to the absolute count, the percentages of different immune cells also varied in our pediatric cohorts in comparison to that of Europe, Africa and USA [8,14,16,18]. These data collectively indicate that each immune cell subset in different age groups of children varies with the ethnicity and is influenced by the geographical region. The lymphocyte subsets are known to vary with the time of collection, use of different

WHAT THIS STUDY ADDS?

- Reference ranges are provided for different lymphocyte subsets in pediatric population from different geographical locations in India.

equipments, procedure for estimation, and the time between the collection and testing [8,9]. Hence, to minimize the variation within the laboratories, proper quality control measures were taken such as use of standard procedure, sample collection in the forenoon hours at all the study sites to avoid diurnal variation, and uniformity of equipment and reagents across the sites. This pediatric cohort did not show significant differences between the sexes, as also observed in other studies [10]; although, reference ranges for the CD4 count and percentages in Indian adults were significantly higher in women [2].

India is a geographically heterogeneous country, hence it was important to assess whether the reference ranges differ in different geographical regions. Our study showed significant differences between the region-wise ranges (mostly East and South) in various parameters in different age groups. These differences might be due to the environmental, genetic or nutritional factors [19-21]. Since this data could not be obtained, these observations need to be confirmed on the larger sample size from the specific age-groups. Moreover, the established ranges could be reconfirmed on a small subset from time to time as described earlier [18]. One of the limitations of our study is insufficient samples available in some regions for children belonging to groups II and III. This could be the due to less number of babies coming for DPT immunization during the study period. It would have been interesting to examine the activation and functional profile of these cells; however, due to the limitation of the parameters that can be tested by the available flow cytometer, it could not be evaluated but could be an important area of future research.

In summary, this study provides reference values for different lymphocyte subsets in Indian children of varying age groups. Age was the only important variable affecting the counts, and sex and geographical distribution did not prove to be significant variables. This data can find application in immune system evaluation of children of Indian origin irrespective of sex, geographical distribution and ethnicity. These age related reference ranges will be helpful to assess the immune defects, and suppression/absence of one or more immune functions in Indian children with primary and secondary immunodeficiencies as well as in autoimmune diseases.

Note: Supplementary material related to this study is available with the online version at *www.indianpediatrics.net*

Ethics clearance: ICMR-NARI Ethics Committee; No. NARI/Age Lymphocyte subsets/10-11/100, dated 22 June, 2010.

Contributors: MT: Study design, data analysis, preparation and review of manuscript; VS: analyzed the data, drafted and reviewed the manuscript; NJ,VR,AD,SS,NS,RM,AS,MC,MM: study design, execution of study, patient information, data analysis, manuscript review (at different sites); SS: data analysis, manuscript preparation and review; AM: study design, data analysis manuscript review; VM: study design, execution of the study and review of the program. All authors approved the final version of manuscript, and are accountable for all aspects related to the study.

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Supplementary Table I Lymphocyte Subsets in Healthy Indian Children From Different Geographical Regions (*N*=1104)

Region	*Group I:Newborn / cord blood*				*Group II: 6- 32 wk*				*Group III: 9 -10 mo*				*Group IV : 15-18 mo*			
	North (n=51)	*East (n=50)*	*West (n=44)*	*South (n=49)*	*North (n=39)*	*East (n=23)*	*West (n=5)*	*South (n=0)*	*North (n=24)*	*East (n=49)*	*West (n=3)*	*South (n=49)*	*North (n=28)*	*East (n=46)*	*West (n=11)*	*South (n=47)*
CD3+cells Absolute counts	2321	3241.5	2713.5	2676	3001	4326	NA	NA	4148	4805	NA	4442	3728	4143	NA	3484
% CD3+cells	62	66	65.5	67	63	64	NA	NA	64	60	NA	64	59	60.5	NA	69
CD4+cells Absolute counts	1497	2104	1871.5	1744	2081	2770	NA	NA	2498.5	2977	NA	2840	2108	2476	NA	2036
% CD4+cells	36	43	45	44	42	46	NA	NA	44	38	NA	40	38	37.5	NA	38
CD8+cells Absolute counts	789	1048.5	712.5	889	854	1059	NA	NA	1272	1540	NA	1357	1363	1425.5	NA	1250
% CD8+cells	20	22	18	20	17	18	NA	NA	20	21	NA	20	21.5	21.5	NA	25
CD4/CD8 ratio	1.72	1.91	2.53	2.18	2.22	2.52	NA	NA	2.22	1.89	NA	1.96	1.80	1.79	NA	1.52
CD19+cells Absolute counts	797	795	793.5	459	1392	1727	NA	NA	1634	2167	NA	1836	1771	1746.5	NA	891
% CD19+cells	21	17	19.5	13	27	27	NA	NA	27	30	NA	25	30	29.5	NA	19
CD16/56+ cells Absolute counts	426	517.5	413	648	490	453	NA	NA	452	425	NA	407	442	342.5	NA	355
% CD16/56+cells	9	11.5	11	18	8	6	NA	NA	7	7	NA	6	6	6	NA	7

Region	*Group V 19 mo-5 y*				*Group VI 5 y-12 y*				*Group VII 12 y-18 y*			
	North (n=44)	*East (n=54)*	*West (n=49)*	*South (n=50)*	*North (n=55)*	*East (n=44)*	*West (n=52)*	*South (n=59)*	*North (n=40)*	*East (n=21)*	*West (n=38)*	*South (n=50)*
CD3+cells Absolute counts	3129	3380	3141	2869	2350	2539	2273	2264	1870	2051	2128	2024
% CD3+cells	64.5	62	62	66.5	65	68	65	70	65	72	66.5	66
CD4+cells Absolute counts	1797.5	1996	1791	1707	1236	1327	1154	1364	955	1050	1068	1150
% CD4+cells	39	36.5	36	40	34	36.5	36	40	34.5	36	35	37
CD8+cells Absolute counts	1091	1156.5	1109	1045	876	950.5	892	905	675	785	757.5	819.5
% CD8+cells	23	21	21	25	25	25.5	26	27	25	29	25	25.5
CD4/CD8 ratio	1.75	1.87	1.81	1.61	1.42	1.42	1.35	1.48	1.47	1.26	1.34	1.46
CD19+cells Absolute counts	1171	1358	1207	962	667	697.5	623	641	499	322	528	630
% CD19+cells	23	26.5	25	23	19	18.5	19.5	19	18	14	19.5	20
CD16/56+ cells Absolute counts	357	403.5	334	259.5	421	360.5	377	325	324	257	355.5	349.5
% CD16/56+cells	7	7.5	8	7	11	9	10	9	12.5	8	12	11

The table shows the region-wise median values of each parameter and each age group. These values were compared with the respective overall median values (mentioned in table II for the difference. There was a significant difference between the region wise values and overall values (as indicated by the cells filled with gray color) denotes significant difference. The value of $p<0.05$ was considered to be significant. NA indicates 'not applicable' due to insufficient number of children in the group.