ORIGINAL ARTICLE

Immunophenotyping of Peripheral Blood Lymphocytes and its Association with Age in Healthy Malay Children

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ABSTRACT

Introduction: Numerous research demonstrated the different distributions of lymphocyte subsets in each population. Most studies were performed in Caucasian population. To date, no studies on normal lymphocyte subsets have been conducted involving the children population in Malaysia. Therefore, we conducted this study to determine normal lymphocyte subsets in healthy Malay children (major ethnic group in Malaysia) and to identify the association between age and each of the lymphocyte subsets (CD3+, CD4+, CD8+, CD19+ and CD16/56+). **Materials and methods:** An approximately 500 µl of blood was taken from 93 healthy Malay children and was subjected to flow cytometry immunophenotyping technique. The subjects were categorised into three age subgroups, namely 0 to 1, 1 to 6 and more than six years old. The mean comparison was done using descriptive analysis and the association of lymphocyte subsets with different age groups was identified using one-way ANOVA. **Results:** The results showed that the CD3+, CD4+, CD16/56+ and CD19+ subsets were significantly different in all age groups with p-values <0.05. The highest percentage and absolute count of CD3+ and CD4+ were recorded in the 0 to 1 year age group which the values significantly decreased with age. **Conclusion:** Our study may provide an overview and preliminary guidance on the pattern of lymphocyte subsets distribution in the Malay children's population in Malaysia. For reference range it is recommended that further research is done involving bigger sample size, dividing the samples into more age groups and by sex as well as involving other ethnic groups in Malaysia.
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INTRODUCTION

Lymphocyte subsets are crucial in the human immune system for various reasons, including disease detection and understanding lymphocyte maturation and age-related changes in the phenotype (1,2). Numerous studies have reported on the importance of lymphocyte subsets information in diagnosing and monitoring several diseases. For example, lymphocyte subsets reference was established in several studies to identify and classify some immunodeficiency disorders as well as to monitor infectious diseases, including tuberculosis and malaria (3,4). Furthermore, CD4 T cells are found to be useful in measuring HIV progression, monitoring antiretroviral therapy, and in prophylactic treatment of opportunistic infections. Several studies were also performed to establish a reliable reference range for peripheral blood lymphocyte subsets in different countries and populations (5–7). These studies demonstrated the distribution of normal lymphocyte subsets were different for certain age and population groups.

In the study by Sagnia *et al.* (2011) the B-cell values of the healthy , HIV -negative children in Cameroon were found to be higher than the reported values from developed countries (3). Several studies also demonstrated different distributions of lymphocyte subsets by racial groups (8,9). A study done by Tsegaye *et al.* (2003) demonstrated that Ethiopians have significantly lower CD4 and a higher CD8 ($p<0.001$) compared to Caucasians (10). Similarly, Embree *et al.* (2001) showed that the CD4 values in Kenyan children were significantly lower ($p<0.001$) than those of the Caucasians (11). In addition, Motley *et al.* (1996) found that black babies had significantly higher NK and naïve CD4 cells ($p<0.001$) compared to white

neonates (12). There are different distributions of normal lymphocytes subsets among various racial populations.

Several other studies also showed that the total number and percentage values of most subpopulations of lymphocytes vary or differ significantly with age. For instance a study by Tsegaye *et al.* (2003) found that neonates and children had substantially higher CD4 T cells compared to adults (10). Another study that compared between different age groups (1 month to 13 years) of the Arab population showed decreases in the absolute numbers of leukocytes, total lymphocytes, T, B, and natural killer (NK) cells. As reported in the study while the proportion of T cells increase with age, and the B cells percentage was declined and the NK percentage was unchanged (6). Meanwhile the study of healthy urban-dwelling infants, children, and adolescents in the United States showed cell-surface marker analysis; which demonstrated that age was a significant variable in the lymphocyte subsets analysis (13).

In Malaysia, Malays are the major ethnic group, however so far, no studies have been conducted to explore the distribution of normal lymphocyte in this population especially involving children. Therefore, this study aimed to determine normal lymphocyte subsets in healthy Malay children and to identify the association between age and each of the lymphocyte subsets (CD3, CD4, CD8, CD19 and CD16/56). Data obtained in this study can provide an overview and preliminary guidance on the distribution of lymphocyte subsets in children with different age groups which in future may be used as a guidance for interpretation of disease related changes in the major population ethnic group in Malaysia and also in other countries with similar ethnic groups.

MATERIALS AND METHODS

Study design and population

The study samples were obtained from several sources in which the subjects included the staff's children and other children who either went to the clinics for routine daily checkup or admitted to the ward for minor surgical procedure at the Advanced Medical and Dental Institute, USM, Hospital Kepala Batas and Hospital Seberang Jaya. In general Malay children with good health condition were recruited in this study including healthy children who attended the clinics for routine growth and development monitoring and also prior to receiving their scheduled vaccination. Other than that, those who were admitted to the hospitals for pre-surgery screening due to benign diseases such as inguinal hernia repair, lumps and bumps and circumcision were also involved in this study. Additionally, the subjects fulfilled the inclusion and exclusion criteria in which at the time they were being recruited, they were not known to have any chronic disease, haematological disease, syndromic or not on immunosuppressive drugs. The study was approved by the Research and Ethics Committee (School of Medicine, Universiti Sains Malaysia (USM), Kelantan Health Campus, JEPeM Code:USM/JEPeM/17080373) and the Medical Research Ethics Committee (Secretariat of National Institute of Health, NIHSEC, NMRR-17-2765-37913- (IIR).

Inclusion and exclusion criteria

The inclusion criteria included Malay children, regardless of their gender, who are healthy aged from newborn until 17 years old with normal levels of blood pressure (systolic pressure/diastolic pressure mmHg) and pulse rate (60 to 100 beats per minute), have a proportionate height-to-weight ratio, as well as no cough, fever, infection, or history of immunological or autoimmune disease and not taking any medication. Malay is defined as up to three generations above with no history of mixed ethnicity marriages. As for exclusion criteria those who were ill, exhibit any fever or with sources of upper respiratory tract infection (URTI), urinary tract infections (UTI), gastrointestinal tract (GIT) and central nervous system (CNS) symptoms within five days prior to the sample collection were excluded from the study. Additionally, children with medical conditions that can affect their immune systems, such as autoimmune disease, HIV/AIDS, allergies, neoplasms, endocrine disorders, diabetes mellitus, as well as acute and chronic infections were also not eligible to participate in this study.

All children underwent clinical evaluation. The demographic information including gender, date of birth, family history, consanguinity and laboratory data were recorded using a questionnaire in order, to determine if the subjects had any immunological or hematological abnormalities or had any medication. The age of the subjects ranged between 0 and 14 years. The study group (n=93) were categorised by three subgroups based on age: 0-1 year (n=31), 1-6 years (n=31) and >6 years (n=31). Written informed consent was obtained from all parents.

Sample and staining preparation

An approximately 500 µl of blood was taken from each subject via venipuncture by trained staff nurses and medical officers. The sample blood was placed in tubes containing EDTA before being transported to the laboratory at ambient temperature. The blood was then processed within six hours of venesection. A total of 20 µl of each monoclonal antibody (BD Bioscience, USA) was mixed with 50 µl of whole blood in BD Trucount tube (BD Biosciences) and gently vortexed. The tube was incubated for 15 minutes at room temperature in the dark. Next, 450 µl of diluted FACS Lysing solution was added (10x dilution, BD Biosciences) into the mixture vortexed and incubated for another 15 minutes at room temperature in the dark. The cells were ready to be analysed on the flow cytometer after running the BD

7 Colors setup beads.

Antibody for flow cytometry

Monoclonal antibodies were used in this study for the enumeration of T, B and NK cells. The multicolour antibody reagents used for staining were as follows: CD3 FIT C / CD4 APC / CD8 PE / CD19 APC / CD 16+CD56 PE (NK cells). All monoclonal antibodies were purchased from Becton Dickinson, Mountain View, CA. The single platform lymphocytes were gated based on their forward and side scatter properties and all cell subpopulations were acquired using Clinical Canto Software. The overall percentage and absolute counts of all the lymphocyte subsets ($CD3^+$ T cells, CD3-CD19+ B cells, $CD3^+$ $CD4^+$ T helper cells, $CD3^+$ $CD8^+$ T suppressor/ cytotoxic cells, $CD3^-$ $CD16^+$ $CD56^+$ natural killer cells) were investigated. All the flow cytometric analyses were performed through BD FACSCanto™ II flow cytometry equipped with three lasers (blue/ red/ violet), and eight fluorescence detectors. The MultiTEST reagents employed fluorescence triggering, allowing direct fluorescence gating of the lymphocyte population. By using the Trucount tube, the absolute count and overall percentage of the lymphocyte subpopulation or subsets were calculated. The total lymphocyte population was identified based on forward and side scatter characteristics (FSC and SSC, respectively). Figure 1 shows the gating strategy with a dot plot. The plots were generated from this lymphocyte region by combining two antigens expressed on the cell surface which were separated by quadrants, allowing identification and determination of the lymphocyte subsets. Internal quality control was performed through the daily checking of the cytometer optical detector voltages and by aligning the laser and fluid system using the BD FACS™ 7-Color setup beads according to the manufacturer's guidelines.

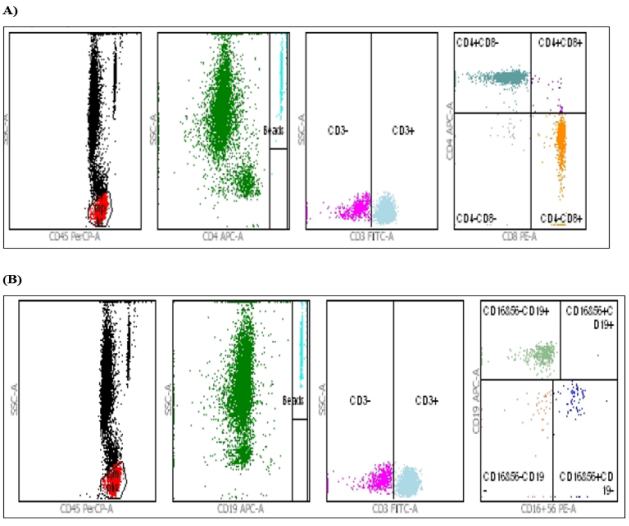

Figure 1: The flow cytometry gating strategy of the lymphocyte subsets. The lymphocyte population was selected from total cells as determined by the forward scatter/side scatter plot and the CD4+ T cells were selected from the lymphocyte population. (A) The Trucount T cells were gated as CD3/CD8/CD45/CD4 and (B) trucount gated for NK and B cells as CD3/CD16+56/CD45/CD19.

Statistical method

Statistical analyses were carried out using SPSS Version 26 for Windows. One sample Kolmogorov – Smirnov test was used to check the Gaussian distribution, since not all normal distribution variables are observable. By assuming a Gaussian distribution for the observation, reference intervals were established using the parametric analysis guided by Lab Statistics book (14). In establishing the reference range, a simple calculation of mean ± 2SD was done. The mean comparison of analysis was carried out using the descriptive analysis and the association of lymphocyte subsets with age was identified using one-Way ANOVA. A two-sided P value < 0.05 was considered statistically significant.

RESULTS

Out of the total 105 healthy Malay children were recruited in this study, only 93 met the criteria as explained in the Materials and Methods section and thus were included for further analysis. From the selected subjects, 22 were females (23.66 %) and 71 were males (78.89 %). The age of the subjects ranged between 2 dayst to 14 years old with a mean age of 49.13 months (range: 0.06-192.5). The subjects were categorised into three subgroups based on age: 0-1 year (n=31, male=23 and female=8), 1-6 years (n=31, male=28 and female=3) and >6 years (n=31, male=20 and female=11).

The overall percentage and absolute count of each lymphocyte subset for the three groups are displayed Table I. The absolute valtues are expressed in number of cells per microliter, while the percentages refer to the relative frequencies of each subpopulation concerning the total lymphocytes. As shown in Table I and Figure 2 the mean values of the absolute counts of total lymphocyte CD3 T cells and CD4 T cells declined sharply across the age groups. Both CD3 and CD4 T cells percentages and absolute counts were the highest during the first year of life and gradually decreased in older children. Meanwhile for CD8 Tcells and CD16 NK cells, the highest mean values of the absolute counts were observed in the group of children aged more than 6 years old. For CD19 B cells , children aged between 1-6 years indicated the highest mean value of absolute count with 1221cells/ul (Table I).

Table I: The absolute count and percentage of the lymphocyte subsets in each age group

Mean value of the cell count (cell/mm³) ± 2SD (range) for each group						
Cell Type	**0 – 1 Year, n=30 Abs Count (µL)**	**Percentage (%)**	**1 – 6 Years, n=30 Abs Count (µL)**	**Percentage (%)**	**>6 Years, n=30 Abs Count (µL)**	**Percentage (%)**
CD3 T CELLS	3894 (1939-7260)	76 (55-87)	3482 (1555-6707)	63 (51-74)	2550 (904-5049)	66 (47-80)

CONTINUE

Table I: The absolute count and percentage of the lymphocyte subsets in each age group (CONT')

Mean value of the cell count (cell/mm³) ± 2SD (range) for each group						
Cell Type	**0 – 1 Year, n=30 Abs Count (µL)**	**Percentage (%)**	**1 – 6 Years, n=30 Abs Count (µL)**	**Percentage (%)**	**>6 Years, n=30 Abs Count (µL)**	**Percentage (%)**
CD4 T CELLS	2752 (1002-5496)	53 (32-70)	1832 (949-3484)	34 (18-48)	1163 (356-1882)	31 (10-44)
CD8 T CELLS	1121 (420-2439)	22 (14-32)	1416 (454-3194)	26 (14-42)	1215 (373-2919)	32 (21-53)
CD19 B CELLS	812 (203-2883)	15 (4-37)	1221 (230-2516)	22 (14-39)	624 (326-1741)	17 (8-31)
CD16/ CD56 NK CELLS	389 (86-1050)	7 (2-19)	744 (198-1994)	13 (3-28)	574 (202-1452)	15 (8-39)
CD4: CD8 RATIO	1.2-4.4	1.2-4.4	0.6-3.0	0.6-3.0	0.2-1.8	0.2-1.8

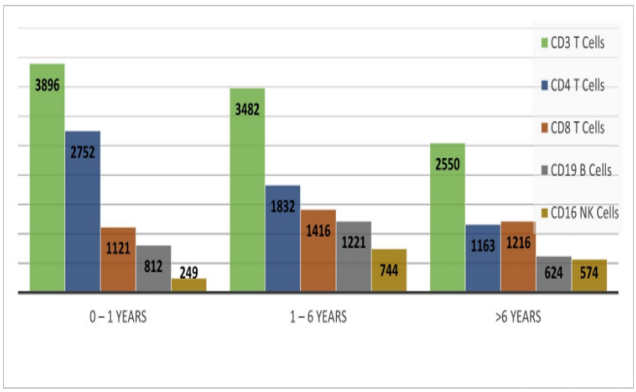

Figure 2: Mean value of the absolute count of lymphocyte subsets in each age group.

Analysis of one-way ANOVA results in Table II, revealed significant differences of all lymphocyte subsets across all age groups (p=<0.05). Further analysis using Dunnett's T3 post-hoc for multiple comparisons was done to determine the most significant differences. The results indicated a significant mean difference in the CD3+ T cells among all the age groups except comparison between 0-1 year and 1-6 years which showed non significant differences between these two groups. For CD19 B cells, significant results were recorded for two pairs, i.e between the 0-1 year and 1-6 years age groups, and between 1-6 years >6 years age groups. Meanwhile the analysis for the CD16 NK cells showed significant between 0-1 years with 1-6 and >6 years age groups. For the ratio CD4:CD8, all pairs of the age groups showed significant mean differences, whereby the highest mean difference recorded for this lymphocyte was 1.61, i.e between the 0-1 year and >6 years age groups.

Table II: Mean comparison of the lymphocyte subsets in different age groups (n=93)

Subsets	Age Groups	Mean	SD	F-stat	p-value*
CD3T	0-1 year	3895.91	1245.28	10.89	<0.001*
	1-6 years	3481.64	1344.17		
	>6 years	2549.56	839.58		
CD4T	0-1 year	2752.14	1014.25	34.19	<0.001*
	1-6 years	1832.25	731.04		
	>6 years	1162.65	411.21		
CD8T	0-1 year	1120.85	426.66	2.66	0.075
	1-6 years	1416.02	618.88		
	>6 years	1215.92	478.31		
CD19-CD20	0-1 year	812.30	572.50	11.53	<0.001*
	1-6 years	1220.58	591.79		
	>6 years	624.24	267.66		
CD16NK	0-1 year	389.03	249.14	6.73	0.002*
	1-6 years	744.41	528.43		
	>6 years	573.53	308.10		
CD4:CD8 RATIO	0-1 year	2.63	0.92	52.67	<0.001*
	1-6 years	1.40	0.53		
	>6 years	1.03	0.35		

*The mean difference is significant at the 0.05 level

DISCUSSION

This study revealed that the absolute counts of the lymphocyte subsets of the Malays changed significantly from infancy to adolescence (Figure 3). Age has been found to affect the lymphocyte subpopulations in numerous research (8,15). According to the studies by Kokuina *et al.* (2019) and Swaminathan *et al.* (2003), the absolute and percentage values for most lymphocyte markers differ dramatically among children not only by different ages but also by ethnic groups and countries. (16,17). As a result, neither adults nor children of mixed ages can be used to provide the reference values for the lymphocyte subsets in infants and children. Thus, an accurate interpretation may require the development of an age-specific reference for the lymphocyte subsets in each country or population (16,17).

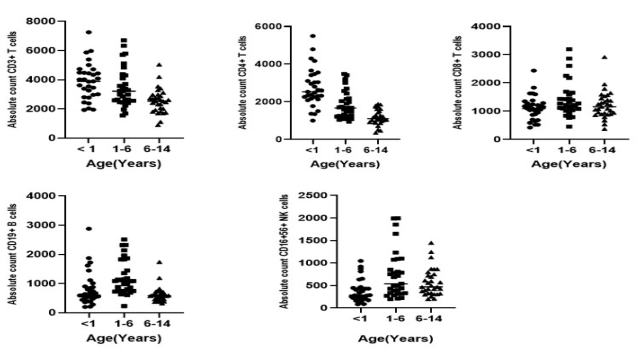

Figure 3: The absolute count of lymphocyte subpopulations among different age groups.

The comparative data analysis between three age groups (Table I) has proven that age is a significant factor affecting the number of counts and percentage of the different factor lymphocyte subsets of the CD3 T, CD4 T, CD8 T, CD19 B and CD16 NK cells. This result is similar to other studies which also reported that most lymphocyte subpopulations had significant differences in the absolute and percentage values as the subject got older (18,19). All of the absolute numbers of leukocytes, total lymphocytes, T, B, and natural killer (NK) cells were all decreased with age, from infants to adults (6,20,21). In this study, the highest composition of the CD3 T cells and CD4 T cells was recorded in the age group of less than one year. As the age increased the numbers of CD3 T and CD4 T cells declined significantly ($p<0.01$). As Palmer (2013) suggested, as age increases the levels of CD3 T and CD4 T lymphocytes may become lower due to the potential degradation of lymphoid cell growth and function at various levels related to lower immune response efficacy, as mainly seen among the elderly (22). With increasing age, a lower potential of hematopoietic cells for lymphoid differentiation also decreases the formation of immune cells.

Nonetheless, our findings revealed that the absolute count of CD8 T cells was surprisingly consistent from young to older age. Other studies demonstrated varying results pertaining to the non-significant of age differences in the cell distributions, some of the results were low during early childhood, whereas others were greater at this stage. In this study, the number of CD8 T cells or cytotoxic T cells increased at one to six years, but the value declined after six years. In the study by Van *et al.* (2009), the absolute number of CD 19 increased shortly after birth to a maximum at 6-12 months and decreased afterward (23). Their result somehow contradicts our results whereby the B cell absolute count was initially low during the first years of life. During the next two to six years, the B cell increases and then decreases in the following ages.

The cytotoxic granular lymphoid cells, known as natural killer (NK) cells, originate from the same progenitor as B and T cells. NK cell are the nonspecific immunity or innate immune system that contributes to the inflammatory environment when an infection is present (24). NK cells also are defined by the ability to kill cancer cells and to identify virus-infected cells. According to Stervbo *et al.* (2015) the total number of NK cells rises with the aging process (25). NK cells are easily activated due to the immaturity of the lymphocyte during early life (26). This is concurrent with our result where the numbers of NK cells were significantly different by age group ($p=0.02$) in which NK absolute count was initially low during the first year but then increase between one to six years and finally decreased after more than six years.

In this study, CD3 and CD4 T cell percentages were lower than those of other earlier European and American research (8,27). Fascinatingly, our results align with those of previous Asian research (28–30). Compared to other Asian populations, children under the age of one have a larger percentage of CD3 T Cells (28,30–32). Moreover, the percentage of CD8 T cells obtained in this study also significantly differed from previous studies, except for a Saudi Arabian study (6) which reported a greater percentage value of the cells above six years old, as similarly noted in our study. Our result on the increasing percentage value of CD8 by age groups is also consistent with several other studies.(9,10,30,33).

Comparing to other Asian populations the percentage of NK cells obtained in this study was also similar to that of Chinese children in China (5,32) but greater than that of Thai children in Thailand (30). It is also observed that our reported percentage of NK cells was higher than of the in children over five years old in Europe and America (9,10,13,34). Several studies suggested that infections such as dengue, could be a potential cause of the increased frequencies of NK cells with advancing age, this may also explain the higher NK percentages in the Malay population since dengue is among the common infectious diseases in Malaysia (35,36).

The influence of ethnicity and certain sociodemographic characteristics on the different distributions of the lymphocyte subsets has been proven by several authors (16,32,37). Overall, our study is in agreement with their result on the differences in lymphocyte subsets compared to other populations in Asia, Europe, Africa, and America. This could be due to a variety of biological and sociodemographic factors such as age, gender, ethnicity, and environmental factors like infection exposure, air pollution, and lifestyle (38–40). Thus, specific ranges of lymphocyte subpopulations should ideally be identified by economically developed populations and updated through periodic reevaluation, considering the influences of environmental and sociodemographic factors that are constantly changing over time.

CONCLUSION

The absolute count and percentage values of the lymphocyte subsets in Malay children differed from those obtained in other countries. Our finding identified significant association of the CD3T, CD4T, CD19-CD20, CD16NK and the CD4:CD8 ratio lymphocyte subsets with all the age groups (0-1 year, 1-6 years, and more than six years) with p-value obtained less than 0.05. The absolute values of the total lymphocyte and CD3+ CD4+ T lymphocytes were higher in younger participants. Our study may provide an overview and preliminary guidance on the pattern of lymphocyte subsets distribution among the Malays children population in Malaysia. However, considering the limitation of this study in terms of small sample size and number of age groups, large scale-studies are still needed to confirm our findings. As a

reference range, it is recommended for future studies to further explore the case of lymphocyte subsets and the associating factors by involving with bigger sample size, dividing the samples into more age groups and by sex as well as involving other ethnic groups in Malaysia. It is therefore important to establish a comprehensive age-specific reference value of the lymphocyte subsets for appropriate clinical evaluation and treatment of patients with immunological and hematological disease among the population in Malaysia as well as other countries with similar socioeconomic, cultural, and environmental conditions.

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