Normal ranges for peripheral lymphocyte subsets in healthy adults' Malay population in Malaysia

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ABSTRACT

INTRODUCTION: Individual's immune system needs to be assessed in different diseases. Lymphocytes are the main cellular player.

OBJECTIVE: The aim was to establish the normal ranges for T–lymphocyte subsets, B–lymphocytes and natural killer (NK) cells in peripheral blood of healthy adults' Malay individuals in Malaysia. Secondly, was to compare the results with previous studies in Malaysia, with Caucasians, and Ethiopians. Thirdly, was to assess the difference in percentage values of these lymphocytes in different age groups and sex.

METHODS: Dual-platform flow cytometry and automated haematological analyser were used for this study. Eighty-four healthy Malay volunteers, aged 18 years and above, residing in Bertam region at Penang, Malaysia were involved through a cross sectional study.

RESULTS: For the normally distributed parameters the normal ranges were calculated as mean ± 2SD; CD4 was (31.13 ± 14.65) %, CD8 was (31.36 ± 15.14) %. However, for the not normally distributed parameters, the normal ranges were defined as 95% of the area under the distribution curve of the values; CD3 was (16.50–75.00) %, CD19 was (5.10–24.61) %, and $CD16^+$ and/or $CD56^+$ was (6.01–44.59) %. Ranges in counts were also presented.

CONCLUSION: Regarding the gender; females have significantly higher CD4 and lower $CD16^+$ and/or $CD56^+$ percentages. While concerning the age, there were significant differences in all parameters except for $CD16^+$ and/or $CD56^+$ lymphocytes. The percents of CD3 and CD8 declined significantly after age of 40 years, while CD4 and CD19 increased significantly after age of 30, 40 years respectively.

Key words: CD; lymphocytes; Malaysia; normal range; percentages.

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INTRODUCTION

The immune system has important functions in the body. Infectious microbe or carcinogen stimulates the immune system which responds through recruitment of many humoral and cellular effectors.[1] Lymphocytes are the main cellular blood component, which are divided into many subsets; each performs different functions during immune response and each characterized by distinct markers (antigens).[2] This project utilized these markers to measure the corresponding lymphocyte subsets in the peripheral blood, through using specific monoclonal antibodies, directed against the corresponding specific antigens.

Clinicians need to assess the immune status of the individuals, interpret laboratory results, and follow up the disease status then, subsequently, choose the appropriate treatment strategies. Many studies have been done worldwide to determine the reference ranges of peripheral lymphocytes, they have revealed that the normal range of each subset may differ according to age,[3] sex,[2-3] race[4] and environmental factors,[5] such as prevailing mycobacterium infection, helminthic infestations, hepatitis and

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poor nutrition,[6] current malaria,[7] dietary patterns,[8] and smoking habits[9] could be the suspected reasons of making a difference in results of studies of the same nature.

The objectives of this article was to establish the normal ranges of the subsets of T-lymphocyte, B-lymphocytes and natural killer (NK) cells in peripheral blood of healthy adults' Malay individuals in Malaysia. Secondly, was to compare the results with previous studies in Malaysia, with Caucasians, and Ethiopians. Thirdly, was to assess the difference in percentage values of these lymphocytes in different age groups and sex.

METHODS

Settings and study design: This study is performed by using a sampling method design through a cross sectional study, to set up the normal ranges and investigate the difference between lymphocyte subsets with age and gender. The study was done during March and April 2011. It was carried out at the Advanced Medical and Dental Institute (AMDI), Universiti Sains Malaysia (USM), Cluster of Transfusion Medicine, No. 1 _ 8, Persiaran Seksyen 4\1, Bandar Putra Bertam 13200, Kepala Batas, at the region of Bertam at the mainland of the state of Penang, Malaysia.

Ethical issues: The approval from the Ethical Committee of Research, and informed consent from the participants were obtained in accordance with the Declaration of Helsinki.

Selection and Description of Participants: The 84 volunteers were healthy Malay volunteers from the region of Bertam at the mainland of the state of Penang, Malaysia. They were gender matched (42 males and 42 females), and they were assigned according to their ages into three groups (first; 20- 29 years, second; 30-39 years, and third age group; 40 years and above).

Inclusion criteria:

1. Healthy individuals.
2. Age ranging from 18 years and above.
3. Malay race (The individual, his parents and his grandfather and grandmother, all are Muslims, Malay, speak Malay, culturally practice Malay and live in Malaysia or Singapore, according to Article 160 in Malaysian Law).
4. Males and females.

Exclusion criteria:

1. Persons who have taken any medications during the last two weeks prior to the sampling.
2. Persons with any chronic or acute infections (viral or bacterial)
3. Persons with chronic diseases including malignancy
4. Pregnant women.
5. Abnormal high total lymphocytes count (> 4.0 x 10^3/µL).[11-12]

Technical Information:

Primary outcomes: The percentage and count of each T-lymphocyte subset; CD4, CD8, CD19, NK and $CD2^-CD3^+$ cells, in general, then with regard to gender and age. This is done by lymphocytes Immunophenotyping, using Flow cytometer (BD FACSCanto II). It was performed by the researcher, at the Immunology Laboratory at the institute – Lot 10.

Secondary outcomes: Complete blood count (CBC) with differential white blood cell (WBC) counts were the secondary outcomes. This is done using Full Blood Counter (Sysmex XE 5000 automated haematological analyser). It was performed by the researcher and the staff nurse at the clinic, Haematology Laboratory in Pusat Klinik.

Procedure: A demographic and health questionnaire was achieved via an interview conducted by a clinical physician before drawing the blood samples between 9 to 11 am and was collected into 2-ml EDTA tubes.

The subjects were categorized into three groups according to their ages; first group: (18 -29 years), second group: (30-39 years), and third group: (40 years and above).

Analysis of Flow cytometry**:** Peripheral blood lymphocyte phenotypes were performed within 8 hours of the collection time using a four-color flow cytometry analysis. All the reagents and consumables were purchased from BD Bioscience Company. Two mixed staining reagents were used; one is the MultiTest CD3 FITC/ CD8 PE/ CD45 PerCP/ CD4 APC; and the second is the MultiTest CD3 FITC/ $CD16^+$ CD56 PE/ CD45 PerCP/ CD19 APC.

For each subject's blood sample, two falcon tubes were used, 1st stained with (CD3, 8, 45, and 4) mixture; the 2nd tube was stained with (CD3, 16/56, 45, and 19) mixture. The staining procedure followed the manufacturer's instructions.

Data acquisition and analysis was performed using FACSCanto II flow cytometry machine and FACSDiva software. An arbitrary forward scatter (FSC) threshold to exclude erythrocytes, platelets, subcellular particles, cell aggregates, and debris was set in data acquisition. Ten thousands events were acquired for each staining tube. In data analysis, lymphocytes were gated using CD45/SSC plot. The lymphocyte subsets were measured as percentages by applying the quadrant gate as in **figure 1 (a, b)**, the lymphocyte subsets appeared as in **figure 1 (b, c, d)**.

Complete blood count (CBC), including an automated differential and total white cell counts, was performed prior to the flow cytometry analysis step, using Sysmex XE 5000 automated haematological analyser (Sysmex Corporation, Kobe, Japan) to get the total lymphocyte count.

Quality control: In order to ensure quality control of the combination of monoclonal antibody reagents used and the performance of the flow cytometer (FCM), the same batch of reagents was used throughout the whole study. The Cytometer Setup and Tracking Beads (CST beads) were used to run day-to-day cytometer performance checks and to reproducibly set up the cytometer. This is performed prior to sample acquisition to maintain the optimum performance of the machine.

Statistics: Analysis was performed using SPSS software (version 18). Descriptive statistics were used to calculate the normal ranges, depending on the results of Wilks-Shapiro test of normality. When the test result was not significant ($p > 0.05$), Gaussian distribution was assumed and the range was defined as (mean ± 2SD), while when the test result was significant, non-Gaussian distribution was assumed and the range was defined as the 95% of the area under

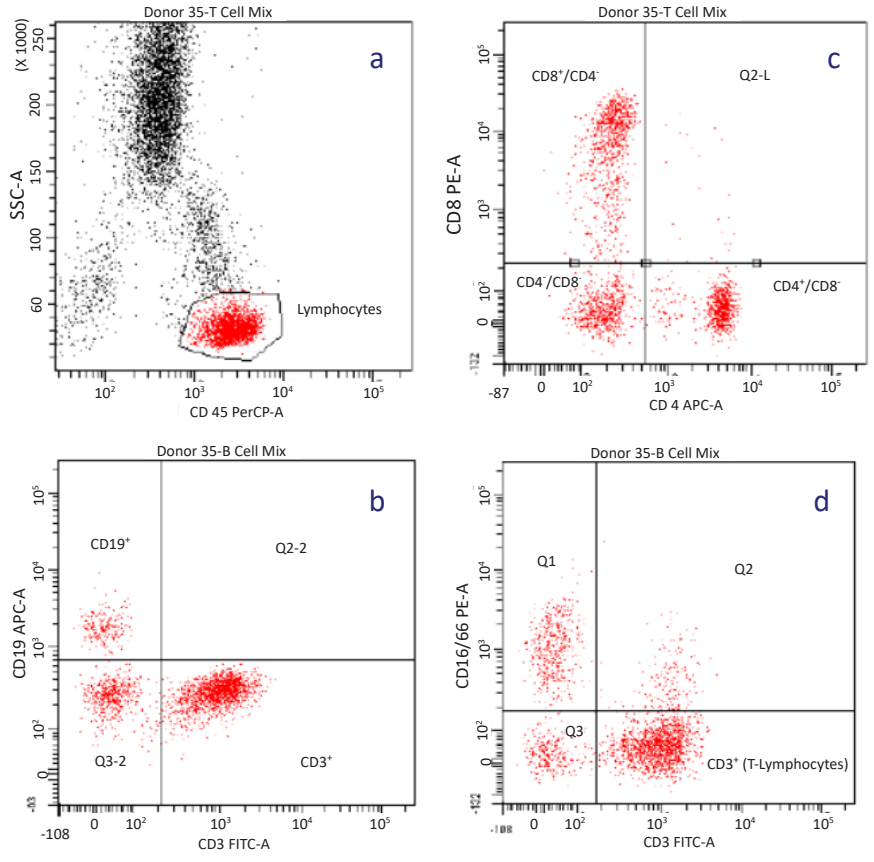

Figure 1: Lymphocyte subsets' measurement by percentage method using quadrant gate plot.
a: The CD45/ SSC plot for isolation of total lymphocytes: putting the lymphocytes in the appropriate position by adjusting the voltages for each channel after using the negative control tube, then gate on these lymphocytes.
b: Plot for isolation of CD3 and CD19 lymphocytes, then measuring their percentages.
c: Plot for isolation of CD4 and CD8 lymphocytes, then measuring their percentages.
d: Plot for isolation of CD16/CD56 lymphocytes, then measuring their percentages.

the distribution curve of the values (from 2.5 – 97.5%). The ranges were calculated in different age groups and in both genders.

The normal ranges in percentages were compared to that obtained from previous studies done in Malaysia by Chin et al,[4] Choong et al,[8] and Dhaliwal et al.[10] For purpose of comparison, the ranges were calculated as (mean ± 1SD). Online t-test calculator was used after obtaining the mean and pooled SD of the other studies. The results in percentages were also compared with the Caucasians',[3] and Ethiopians' results [11] also by online t-test calculator.

To assess the differences in mean values of percentages in both genders, independent sample t-test was used. While for the difference in the three age groups, one way ANOVA was used.

RESULTS

A complete set of parameters were obtained from each participant. The sex distribution of our cohort was equal (42 each). The mean age ± SD was 31.69 ± 8.37 years. The age was categorized into three groups, 46 (54.8%) participants aged 18-29 years, 18 (21.4%) aged 30-39 years, and 20 (23.8%) were 40 years and above. The mean ± SD of the total WBC counts of the subjects were 7.17±1.66x 10^3/µl.

The percents of CD4 and CD8 subsets were normally distributed around the means, while CD3, CD19 and $CD16^+$ and/or $CD56^+$ percents were not. Regarding the counts, CD3 and CD4 subsets were normally distributed, while CD8, CD19 and $CD16^+$ and/or $CD56^+$ subsets were not. Their normal ranges in percentages and in total counts were presented in **table 1**. The CD3 cell count was estimated for the 84 participants, while for the CD4, CD8, CD19, $CD16^+$ and/or $CD56^+$ cells count were measured for only 81 volunteers because three samples did not retain the stain by the monoclonal reagent antibodies, due to technical error.

Ranges in percentages and counts in rela-

Table 1: Normal ranges for lymphocyte subsets' percentage and count

Lymphocyte Subsets	N		Mean±2SD	(95%)*
CD3 Cells	84	Percent †		16.50–75.00 %
		Count ‡§	1.46 (1.02)	
CD4 Cells	81	Percent ‡	31.13±14.65 %	
		Count ‡§	0.78 (0.55)	
CD8 Cells	81	Percent ‡	31.36 ± 15.14 %	
		Count †§		0.26 – 1.68
CD19 Cells	81	Percent †		5.10 – 24.61 %
		Count †§		0.09 – 0.84
$CD16^+/CD56^+$ Cells	81	Percent †		6.01 – 44.59 %
		Count †§		0.13 – 1.21

* For not normally distributed variable, 95 % of the data (2.5–97.5%) was used, † Not normally distributed, ‡ Normally distributed. § (× 10^3 / µL).

Table 2: Normal ranges for lymphocyte subsets' percentage and count in relation to age groups

Age Group	CD3 Cells	CD4 Cells	CD8 Cells	CD19 Cells	$CD16^+/CD56^+$ Cells
20 – 29 years					
Number	46	46	46	44	44
Median (IQR) %	59.70 (14.53)[L]	30.10 (8.10)[L]	34.10 (6.07)[L]	11.45 (5.73)[R]	20.65 (10.60)[R]
Range* (95%) %	40.53–74.30	14.71–40.32	17.47–45.16	5.11–20.06	6.03–40.33
Range (95%) count†	0.72 – 2.44	0.30 – 1.18	0.42 – 1.37	0.09 – 0.58	0.14 – 1.31
30 – 39 years					
Number	18	17	17	18	18
Median (IQR) %	63.15 (16.18)[R]	31.90 (14.45)[R]	32.40 (16.25)[L]	11.00 (6.50)[R]	18.20 (13.12)[R]
Range* (95%) %	48.80–78.90	19.30–50.10	14.40–49.80	6.60–24.80	6.00–34.20
Range* (95%) count†	1.04 – 2.75	0.43 – 1.36	0.33 – 2.02	0.13 – 0.85	0.11 – 0.95
≥ 40 years					
Number	20	18	18	19	19
Median (IQR) %	55.45 (22.10)[L]	34.25 (12.23)[L]	25.15 (12.05)[R]	15.30 (6.30)[R]	22.40 (11.50)[R]
Range* (95%) %	14.10–70.80	11.30–43.10	13.60–42.50	3.60–47.80	10.20–52.00
Range* (95%) count†	0.33 – 2.82	0.20 – 1.64	0.25 – 1.69	0.08 – 1.29	0.32 – 1.21

* (2.5–97.5%), † (× 10^3 / µL), [L] Left skewed, [R] Right skewed.

Table 3: Normal ranges for lymphocyte subsets' percentage in relation to gender

Gender	CD3 Cells	CD4 Cells	CD8 Cells	CD19 Cells	$CD16^+/CD56^+$ Cells
Male					
Number	42	40	40	39	39
Median (IQR) %	56.20(13.83)[L]	29.95(7.72)[L]	33.50(8.35)[L]	10.70(7.00)[R]	24.60 (11.10)[R]
Range[*] (95%) %	16.0–70.77	14.44–43.96	14.44–49.70	3.60–47.80	6.00–52.00
Range[*] (95%) count[†]	0.34 – 2.78	0.28 – 1.25	0.27 – 1.69	0.08 – 1.29	0.11 – 1.34
Female					
Number	42	41	41	42	42
Median (IQR) %	63.10(14.20)[L]	32.80(8.80)[L]	31.00(10.70)[L]	12.60(5.57)[R]	19.10 (7.95)[R]
Range[*] (95%) %	16.34–78.61	11.57–49.81	13.68–43.57	5.92–24.51	9.55–37.78
Range[*] (95%) count[†]	0.36 – 2.74	0.22 – 1.63	0.24 – 2.00	0.11 – 0.84	0.17 – 0.89

[*] (2.5–97.5%), [†] ($\times 10^3$ / µL), [L] Left skewed, [R] Right skewed.

tion to age groups were presented in **table 2**. All the data were skewed, thus normal distribution was not assumed, so normal ranges were defined as 95% of the area under the distribution curve of the values. Similarly, the normal ranges in each gender were presented in **table 3**.

DISCUSSION

Normal ranges of the subsets of peripheral lymphocyte in the whole subjects as well as in both sexes and in different age groups were generated and the results expressed in percentages and total counts.

The current study focused on the normal ranges of Malays only, while the previous three studies carried out in Malaysia in the nineties[4, 8, 10] targeted the whole Malaysian population; Indians, Chineses and Malays. In order to get a rational analysis, only the ranges of the Malay race from those studies were taken in consideration. **Figure 2a** illustrates the differences between the normal ranges obtained from this study with that of previous studies carried out in Malaysia.

The results of this study showed that the normal range of CD3 percentage was significantly lower than those reported in the previous Malaysian studies ($p<0.001$).[4, 8, 10] The trend of the normal range of CD4 percentage was also lower by the same three studies, but only the one reported by Chin et al reached a significant different level.[4] The normal range of CD8 percentage was significantly lower than that reported by two studies ; Chin et al. and Choong et al.[4, 8] Range of $CD16^+$ and/or $CD56^+$ cells percentage was significantly higher than that documented by Chin et al and Choong et al. ($p<0.002$), while the range of CD19 percentage of this study was insignificantly different from the findings of the three studies.

These differences may be to some extent resulted from difference in age ranges between the current and the past three studies. In the current study, the minimum age was 21 years and the maximum was 50 years and among those, only two subjects were 50 years old. However, the age range was 13 – 68 years in Dhaliwal et al study,[10] 20 – 79 years for Chin et al study, and 18 – 71 years for Choong et al. This narrow age range of the subjects of the current study, having more participants below the age of 30 years, and the inequality of age distribution might led to the discrepancy between normal ranges obtained from different studies, and made the current cohort sample not representative for the whole Malay population, considering that most of the significant changes occur after the age of 50.[7]

The lag period between drawing of the blood sample and processing the sample is an additional factor.[10] In the present study, the turnaround time between blood collection and its processing was around one to two hours and the analysis was carried out on the same day. However, in Dhaliwal et al's study, the time elapsed was six hours, and they used cell fixation in 1% formaldehyde, and kept the samples overnight before analysis. For Chin et al's study, formaldehyde was also used and analysis was performed within 24 hours of preparation. Choong et al also used formaldehyde fixative.

Among other projected reasons, is the WBC counting method. In the current study, the XE

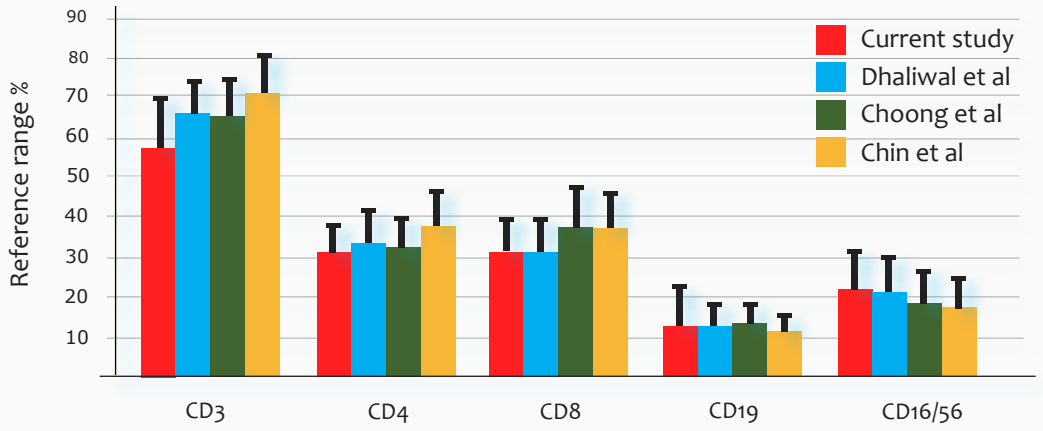

Figure 2a: Differences between the normal ranges of this study with the previous studies on Malays.

5000 was used, while Dhaliwal et al used the model T 540 Coulter Counter, Choong et al used JR Coulter Counter model and obtained the percentages of lymphocytes from manual differential count of 100 WBC on Wright stained blood films. The variation in WBC counts can be overcome with single-platform FCM.[12] Bohler and colleagues[13] compared the results of single and dual- platform FCM and found that the latter underestimates the percent of CD16 and/or $CD56^{+}$ cells, overestimates the percents of CD3, CD8 while yielded comparable percents of CD4 and CD19 with that of single- platform FCM. Other possible causes are the staining reagents and flurochromes, flow cytometer setup and performance status, data acquisition, and analysis (gating strategies and absolute counting).[6, 10, 14-15]

In addition, environmental factors such as prevailing mycobacterium infection, helminthic infestation, hepatitis and poor nutrition,[6] current malaria,[7] dietary patterns,[8] and smoking habits[9] could be the suspected reasons of making a difference in studies of the same nature.

However, the differences in sample processing, suboptimal lymphocyte gating that uses light scattering (FSC and SSC) and (CD14 – CD45

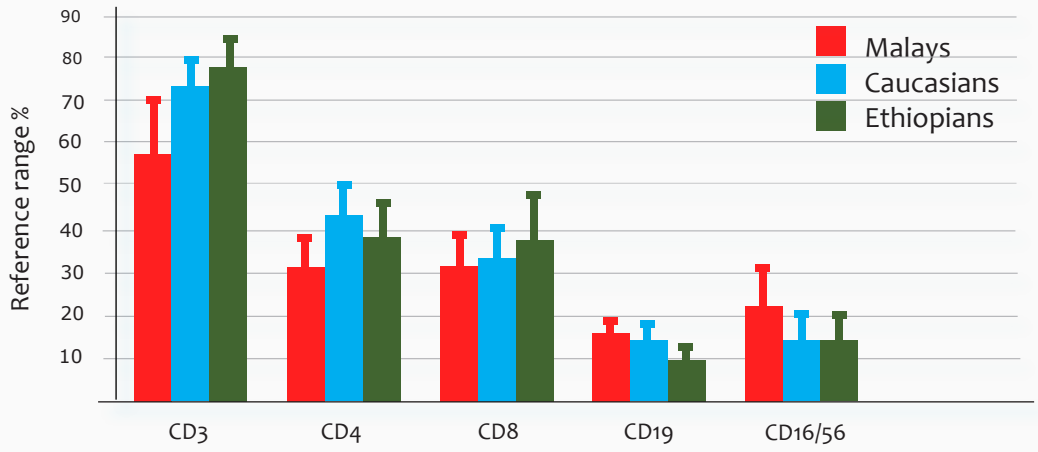

Figure 2b: Differences between normal ranges of Malays, Caucasians and Ethiopians

strategy), and variation in WBC counts were the three major technical causes of the inter laboratory variability.[16]

Recent implementation of guidelines and use of CD45/SSC for gating, due to accessibility of more flourochromes, and the adoption of lyse instead of Ficoll method for sample preparation leads to less inconsistency between different laboratories.[12, 17-18] While single-platform FCM eliminates the discrepancy of WBC counting.[12]

For comparison of the Malay's reference ranges with that of Caucasians and Ethiopians; the mean of CD3, CD4 percent in Malays was significantly lower than in Caucasians and Ethiopians ($p<0.001$). Malay CD16 and/or $CD56^+$ lymphocytes percent was significantly higher than of Caucasians and Ethiopians. While the mean of CD8, CD19 percent in Malays was insignificantly different from that of Caucasians, but CD8 percent was significantly lower than that of Ethiopians ($p<0.001$), and CD19 percent was significantly higher than that of Ethiopians ($p<0.001$). **Figure 2b** illustrated the differences. This might give the evidence of the important effect of race on lymphocyte subsets.[4]

The influence of age and gender

CD3 Cell Subsets:

No significant difference in CD3 cells percent was found between males and females. This was in agreement with other studies among healthy Malaysian adults.[4] However, the finding was different from other studies where females were reported to have significantly higher CD3 percents than males among adult Caucasians and adult Indians respectively.[3, 19]

Significant difference in CD3 cells percent among the three age groups was found in this study ($p = 0.003$). Younger adults (18 – 29 years and 30 – 39 years) found to have significantly higher percentage of CD3 cells comparing with those 40 years and above ($p = 0.018$), ($p = 0.004$) respectively, while it was insignificant between first and second age groups. The percents of CD3 declined significantly after 40 years of age. This is against Choong et al's conclusion, who observed that age has no significant association with CD3 in Malay race. Findings of the current study does agree with Reichert et al and Jiao et al who found that age significantly associated with CD3 which declined significantly with age.[3, 20] Together with other findings from his work, Jiao gave proof for immunosenescence.[20]

CD4 Cell Subsets

A significant difference in percentages of CD4 cells with gender was reported in this study ($p = 0.020$). CD4 percent in female was higher than that in male. On the contrary, other studies of the same nature highlighted insignificant difference in CD4 between males and females as in Chin et al and Choong et al in Malay race and Tollerud et al[5], Chng et al,[6] and Lee et al[12] in studies among Asian populations. In Choong et al study,[8] the difference between genders was significant only among the Chinese race with Chinese female having higher CD4 and CD4/CD8 ratio than male. Caucasians and Indians females have higher CD4 than males according to Reichert et al and Das et al respectively.[3, 19]

There was significant difference in percentages of CD4 cells with age groups ($p = 0.046$), but verification with post hoc test was not possible. This is in conflict to Choong et al[8] who found that there was no difference in CD4 percent with age among the Malay race. The difference was seen only among Chinese race where increased CD4 with age was observed, while Jiao et al study in China found that CD4 declined significantly with age, which is part of immunosenescence.[20] On Caucasian adults, Reichert with other coworkers found that CD4 significantly different by age.[3]

CD8 Cell Subsets

There was no difference in the percentages of CD8 cells with gender, which is in concordance with Chin et al[4] and Choong et al[8] reports.

Significant difference in percentages of CD8 cells with age was calculated in our study ($p = 0.001$). The difference was significant between first and third age group ($p = 0.001$), and was also significant between second and third age group ($p = 0.018$). While difference between the first and second age group was insignificant. This indicates that CD8 decreased after age of 40 years. This is in conflict with Chng et al report on the Singaporean population,[6] who predicted that CD8 is the only subset which increased significantly with age. Choong et al[8] found no

difference in subsets with age of Malay race, while the difference was seen in Caucasians where CD8 increased with age.[3]

CD19 Cell Subsets

In this study, no significant difference was seen in percentages of CD19 cells with gender. This is in connection with Choong et al.[8]

There was significant difference in percentages of CD19 cells with age (p = 0.007). This difference was observed as a significant increase in CD19 between first and third age group (p = 0.005). While according to Choong et al[8], CD19 had no significant difference with age. However, Jiao et al found that CD19 cells decrease with age as part of immunosenescence.[20]

$CD16^+$ and/or $CD56^+$ Cell Subsets

There was significant difference in the percentages of $CD16^+$ and/or $CD56^+$ cells with gender (P = 0.012), the values in males were higher than in females. This is in conformity with Reichert et al,[3] but in conflict with Chin et al and Choong et al who found insignificant difference in these cells between males and females. In Singaporean population, Chng et al confirmed that there was gender difference in these cell percentages,[6] and they argued that this differences were not clinically important because quantification of NK cell subsets does not play a crucial role in clinical management.

There was insignificant difference in percentages of $CD16^+$ and/or $CD56^+$ cells with age groups in the current study. This is in concurrence with Chin et al, where as it is differing to other studies[3, 20] who found significant increase with age, also in disagreement with Price et al,[22] who found that Malays and Chinese were reported to have increased NK cells with age.

There were some studies used CD16 alone instead of $CD16^+$ and $CD56^+$ to enumerate NK cells, which lead to variation in the percents of NK cells. The CD16 antigen is found on all resting NK cells.[23]

Among the limitation of this study is the small sample size with the narrow age range, bearing in mind that the significant changes occur after the age of fifty.[7] The absence of serological tests for subjects prior to inclusion is another limitation. The lacking of the evidence that whole subject were 100% Malay race was also another obstacle of this research.

CONCLUSION

The findings of this study showed that the reference ranges in percentage of CD3, CD4 and CD8 were lower than the past studies carried out in Malaysia, while that of $CD16^+/CD56^+$ was higher, and CD19 was insignificantly different.

Percentages of CD4, $CD16^+$ and/or $CD56^+$ were high in females, while difference with gender was insignificant in CD3, CD8, CD19 and $CD2^-CD3^+$.

The percent of CD3 and CD8 declined significantly after age of 40 years, while CD4 and CD19 increased significantly after age of 30, 40 years respectively. $CD2^-CD3^+$ cell population decreased significantly after age of 30 years.

The reference ranges found from this study could be helpful in assessing individual's immunity status, interpreting diseases and monitoring changes and improvement related to therapy ; they can be employed for a variety of diseases as immune deficiency diseases, auto – immune diseases, lymphomas and leukemia.

REFERENCES

1. Abbas AK, Lichtman AH, Baker DL, Baker A. Basic immunology: functions and disorders of the immune system. 2009: Saunders Philadelphia.
2. Bartlett JA, Schleifer SJ, Demetrikopoulos MK, Delaney BR, Shiflett SC, Keller SE. Immune function in healthy adolescents. ***Clinical and Diagnostic Laboratory Immunology***, 1998; 5(1):105.
3. Reichert T, DeBruyere M, Deneys V, Tötterman T, Lydyard P, Yuksel F, et al. Lymphocyte subset reference ranges in adult Caucasians. ***Clinical Immunology and Immunopathology***, 1991; 60(2): 190-208.
4. Chin SF, Cheong SK, Lim YC, Ton SH. The distribution of immunoregulatory cells in the peripheral blood of normal Malaysian adults. ***Malaysian Journal of Pathology***, 1993; 15(1):49-52.
5. Tollerud DJ, Clark JW, Brown LM, Neuland CY, Mann DL, Pankiw-Trost LK, et al. The effects of cigarette smoking on T cell subsets: a population-based survey of healthy Caucasians. ***The American Review of Respiratory Disease***, 1989; 139(6): 1446-1451.
6. Chng W, Tan G, and Kuperan P. Establishment of adult peripheral blood lymphocyte subset reference range for an Asian population by single-platform flow cytometry: Influence of age, sex, and race and comparison with other published studies. ***Clinical and Vaccine Immunology***, 2004; 11(1): 168.
7. Jentsch-Ullrich Kathleen, Koenigsmann Michael, Mohren Martin, Franke Astrid. Lymphocyte subsets' reference ranges in an age- and gender-balanced population of 100 healthy adults--A monocentric German study. ***Clinical Immunology***, 2005; 116(2):192-197.

8. Choong ML, Ton SH, Cheong SK. Influence of race, age and sex on the lymphocyte subsets in peripheral blood of healthy Malaysian adults. ***Annals of Clinical Biochemistry***, 1995;32:532.

9. Arnson Y, Shoenfeld Y, Amital H. Effects of tobacco smoke on immunity, inflammation and autoimmunity. ***Journal of Autoimmunity***, 2010;34(3):J258-J265.

10. Dhaliwal JS, Balasubramaniam T, Quek CK, Gill HK, Nasuruddin BA. Reference ranges for lymphocyte subsets in a defined Malaysian population. ***Singapore Medical Journal***, 1995;36:288-292.

11. Tsegaye A, Messele T, Tilahun T, Hailu E, Sahlu T, Doorly R, et al . Immunohematological reference ranges for adult Ethiopians. ***Clinical and Vaccine Immunology***, 1999;6(3):410.

12. Barnett G and Whitby S. Absolute CD4+ T-lymphocyte and CD34+ stem cell counts by single-platform flow cytometry: the way forward. ***British Journal of Haematology***, 1999;106(4):1059-1062.

13. Bohler T, Von Au M, Klose N, Muller K, Coulibaly B, Nauwelaers F, et al . Evaluation of a simplified dual-platform flow cytometric method for measurement of lymphocyte subsets and T-cell maturation phenotypes in the population of Nouna, Burkina Faso. ***Clinical and Vaccine Immunology***, 2007;14(6):775.

14. Gelman Rebecca, Cheng Su-Chun, Kidd Pamela, Waxdal Myron, Kagan Jonathan, et al. Assessment of the effects of instrumentation, monoclonal antibody, and fluorochrome on flow cytometric immunophenotyping: A report based on 2 years of the NIAID DAIDS flow cytometry quality assessment program. ***Clinical Immunology and Immunopathology***, 1993;66(2):150-162.

15. Parker John W, Adelsberg Bernard, Azen Stanley P, Boone Donna, Fletcher Mary Ann, Gjerset George F, et al. Leukocyte immunophenotyping by flow cytometry in a multisite study: Standardization, quality control, and normal values in the Transfusion Safety Study. ***Clinical Immunology and Immunopathology***, 1990;55(2):187-220.

16. Gratama Jan W, Kraan Jaco, Van den Beemd René, Hooibrink Berend, Van Bockstaele Dirk R, Hooijkaas Herbert. Analysis of variation in results of flow cytometric lymphocyte immunophenotyping in a multicenter study. ***Cytometry***, 1997;30(4):166-177.

17. Gratama JW, Kraan J, Keeney M, Granger V, Barnett D. Reduction of variation in T cell subset enumeration among 55 laboratories using single platform, three or four color flow cytometry based on CD45 and SSC based gating of lymphocytes. ***Cytometry***, 2002;50(2):92-101.

18. Whitby L, Granger V, Storie I, Goodfellow K, Sawle A, Reilly JT, et al. Quality control of CD4+ T lymphocyte enumeration: Results from the last 9 years of the United Kingdom national external quality assessment scheme for immune monitoring (1993–2001). ***Cytometry***, 2002;50(2):102-110.

19. Das BR, Bhanushali AA, Khadapkar R, Jeswani KD, Bhavsar M, Dasgupta A. Reference ranges for lymphocyte subsets in adults from western India: Influence of sex, age and method of enumeration. ***Indian Journal of Medical Sciences***, 2008;62(10):397.

20. Jiao Y, Qiu ZF, Xie J, Li DJ, Li TS. Reference ranges and age-related changes of peripheral blood lymphocyte subsets in Chinese healthy adults. Science in China Series C: ***Life Sciences***, 2009;52(7):643-650.

21. Lee BW, Yap HK, Chew FT, Quah TC, Prabhakaran K, Chan GSH, et al. Age-and sex-related changes in lymphocyte subpopulations of healthy Asian subjects: from birth to adulthood. ***Cytometry Part B: Clinical Cytometry***, 1996;26(1):8-15.

22. Prince HE, Hirji K, Waldbeser LS, Plaeger-Marshall S, Kleinman S, Lanier LL. Influence of racial background on the distribution of T-cell subsets and Leu 11-positive lymphocytes in healthy blood donors. ***Diagnostic Immunology***, 1985;3(1):33-37.

23. Kitajima Isao, Maruyama Ikuro, Matsubara Hiroshi, Osame Mitsuhiro, Igata Akihiro. Immune dysfunction in hypophosphatemic vitamin D-resistant rickets: Immunoregulatory reaction of 1[alpha](OH) vitamin D3. ***Clinical Immunology and Immunopathology***, 1989;53(1):24-31.

Abbreviation list: Becton, Dickinson (**BD**), Complete blood count (**CBC**), Cluster of differentiation (**CD**), Ethylenediaminetetraacetic acid (**EDTA**), Flow cytometry (**FCM**), Fluorescent isothiocynate (**FITC**), Forward scatter (**FSC**), Natural killer (**NK**), Side scatter (**SSC**), Standard deviation (**SD**), Statistical Package for the Social Sciences (**SPSS**)

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