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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

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We established a normal reference range for peripheral blood lymphocyte subsets in a multiracial adult population by using single-platform flow cytometry. Further analysis of our cohort showed that the $CD8^+$-cell counts decrease with age, there is a gender difference in NK cell percentages and counts, and there are significant differences in the $CD3^+$-, $CD4^+$-, and $CD19^+$-cell counts between Indians and other racial groups. Overall, our results are significantly different from other published data. This difference further stresses the need for different populations to establish their own reference ranges as these may have important implications for the management of patients with human immunodeficiency virus and AIDS. The use of single-platform flow cytometry will eliminate some of the variability between different study centers, making studies more comparable. This platform should be used for future studies into the effects of age, sex, and race on lymphocyte subsets.

Tremendous progress has been made in the field of flow cytometry in the last 2 decades. With the impetus provided by the human immunodeficiency virus (HIV)-AIDS pandemic, the increase in the number of monoclonal antibodies specific for lymphocyte surface antigens, and advances in instrumentation, data analysis, computer technology, and fluorochrome chemistry, flow cytometry has evolved into the most important tool in the evaluation of immunological status (4). The primary clinical applications of immunophenotyping include the enumeration of $CD4^+$-cell counts in the management of HIV infection (25), the characterization of primary immunodeficiency disorders (18, 26), the evaluation of immune-mediated diseases (5), and the assessment of immune reconstitution following stem cell transplantation (38).

Lymphocyte subset analysis is a crucial element in the management of patients with HIV. The importance of absolute $CD4^+$- and $CD8^+$-T-cell counts and the derived CD4/CD8 T-cell ratio in monitoring the progression of HIV infection (11, 36) and the additional value of $CD4^+$ counts in guiding the initiation of prophylactic treatment of opportunistic infections (20) and in monitoring responses to antiviral therapy (46) are well established. The thresholds used in these guidelines are largely based on studies of Caucasians (6). Early immunophenotyping studies were hampered by differences in preanalytical and analytical methodologies that caused interinstitutional variability. Guidelines now exist for $CD4^+$- and $CD8^+$-T-cell enumeration to minimize these problems (1, 25). The introduction of single-platform flow cytometry using fluidics or microbead technology has further reduced the variability caused by differences in white cell count estimation by hematology counters (2).

Many studies have been conducted to establish a reference range for peripheral blood lymphocyte subsets in different countries. These studies revealed variations in the normal range for lymphocyte subsets according to age (9, 10, 35, 39, 45), sex (3, 22, 32, 33, 34, 40), race (7, 8, 19, 22, 30, 31, 42, 43), and environmental factors (41). The clinical implication is that the thresholds for therapy or AIDS definition may have to be established separately for different populations.

The fact that differences exist even between genetically and environmentally similar populations suggests that differences in analytical methodologies may be the source of these variations. Furthermore, few studies compared different racial groups within the same population. Comparing racial groups from different environments is hampered by a number of confounding factors. As a result, whether a true genetic difference in the compositions of lymphocyte subsets exists is still unresolved. However, comparisons with other studies may still be useful in establishing gender differences and age-dependent variability if there are consistent findings across different studies.

With these considerations in mind, we set out to establish the local reference range for lymphocyte subsets, both percentages and absolute counts, by using established guidelines and a single-platform method. Furthermore, the multiracial composition of our cohort would allow interracial comparisons in a single population. The composition of our cohort might also reduce the confounding effect of environmental differences, dietary patterns, and prevailing infections and provide a more accurate reflection of genetic differences.

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MATERIALS AND METHODS

Subjects. A total of 232 healthy blood donors were recruited for the study. Blood donors were screened with an initial health questionnaire that sought to identify volunteers at high risk for HIV-AIDS infection. Volunteers who had any

TABLE 1. Age distribution according to gender

Age range (yr)	No. of male subjects	No. of female subjects	Total no. of subjects
10–19	6	9	15
20–29	39	43	82
30–39	28	41	69
40–49	21	24	45
50–59	7	9	16
60–69	3	2	5

significant medical illness or who were taking medications were excluded. Volunteers were also required to have their HIV, hepatitis B and C, and syphilis statuses checked by either serological or molecular methods or a combination of both. Only blood donors eligible after this screening process were included in our study.

Blood collection. Whole blood was collected into EDTA Vacutainer tubes and transported to the hematology laboratory immediately. The blood samples were stained and fixed on the same day.

Flow cytometry analysis. Lymphocyte subsets were analyzed on a FACScalibur Flow Cytometer (Becton Dickinson). A single-platform, lyse–no-wash procedure was performed with Trucount tubes (Becton Dickinson) with the following two, four-color monoclonal antibody combinations supplied in the MultiTEST IMK kit (Becton Dickinson): CD3-fluorescein isothiocyanate–CD8-phycoerythrin–CD45-peridinin chlorophyll protein–CD4-allophycocyanin and CD3-fluorescein isothiocyanate–CD16 plus CD56-phycoerythrin–CD45-peridinin chlorophyll protein–CD19-allophycocyanin. The stained blood sample was lysed with a diluted lysing solution, and special care was taken not to expose the stained sample to light. $CD3^+$ T cells, $CD3^+CD4^+$ T helper cells, and $CD3^+CD8^+$ T cytotoxic cells were identified according to published protocols. B cells were identified by CD19 expression, and NK cells were identified by the $CD3^-CD16^+$ and/or $CD56^+$ phenotype.

Statistical analysis. Data were entered and analyzed by using Analyze-IT for Microsoft Excel statistical software. The means and standard deviations (SD) were calculated for each marker. The frequency of distribution of each variable was analyzed. If Gaussian distribution was described by the mean and standard deviation, then the reference range was defined by the mean ± 2 SD. If the result of the Wilks-Shapiro test for normality was significant, the data were considered to have a non-Gaussian distribution, and the reference range was defined as the central 95% of the area under the distribution curve of values (from 2.5 to 97.5%). The distribution of T-cell subsets was compared between population groups by Student's *t* test (sex) or analysis of variance (ages and races). *P* values of <0.05 were considered significant.

RESULTS

A total of 232 healthy blood donors ranging in age from 16 to 65 years were included in the study. Of these, 104 (45%) were male and 128 (55%) were female. The racial mix of the study population was as follows: 184 (79.3%) were Chinese, 22 (9.5%) were Malay, 19 (8.3%) were Indian, and 9 (3.9%) belonged to other races, including Caucasian and Eurasian. The age distribution according to sex is presented in Table 1. The sex and racial mix of our cohort were representative of the general adult population in Singapore, and the reference ranges generated from this study should be applicable to the adult Singapore population.

Table 2 shows the means, standard deviations, medians, and reference ranges for the various lymphocyte subsets generated by our study. On the whole, distribution was non-Gaussian for most parameters, and the reference ranges were defined as the central 95% of the area under the distribution curve. The exceptions were the percentages of $CD4^+$ and $CD8^+$ cells which showed Gaussian distribution, and their reference ranges were constructed by using the mean ± 2 SD.

The data were further analyzed according to sex, race, and

TABLE 2. Lymphocyte subset percentages and absolute-number reference ranges of study population[a]

Parameter	Lymphocyte absolute count	Value for cell type: CD3		CD19		CD4		CD8		NK		CD4/CD8 ratio
		% Positive	Absolute count	% Positive	Absolute count	% Positive	Absolute count	% Positive	Absolute count	% Positive	Absolute count	
Mean	2,375	66.9	1,590	14.9	353	35.6	838	27.2	642	17.2	419	1.43
SD	658	7.7	466	4.9	175	6.3	268	6.9	237	7.5	239	0.56
Median	2302	68.0	1550	14.0	335	35.0	814	27.0	616	16.0	364	1.33
Reference range	1,346–3779	49.0–80.0	796–2,679	7.0–28.0	133–714	23.0–48.2	401–1451	13.4–41.0	243–1206	6.0–37.0	115–1009	0.69–2.83

[a] Absolute counts are given in cells per microliter; $n = 232$.

TABLE 3. Lymphocyte subset percentages and absolute-number reference ranges for males and females in the study population[a]

Parameter	Lymphocyte absolute count	Value for cell type:								CD4/CD8 ratio
		CD3		CD19		CD4		CD8		NK		
		% Positive	Absolute count	% Positive	Absolute count	% Positive	Absolute count	% Positive	Absolute count	% Positive	Absolute count	
Mean ± SD[b]												
Male	2,437 ± 645	66.0 ± 7.5	1,609 ± 454	13.6 ± 3.9	349 ± 213	35.0 ± 6.0	837 ± 262	27.3 ± 6.5	660 ± 231	18.8 ± 7.1	472 ± 247	1.4 ± 0.5
Female	2,325 ± 667	67.5 ± 7.7	1,575 ± 477	15.9 ± 5.4	356 ± 138	36.1 ± 6.6	839 ± 273	27.0 ± 7.2	628 ± 242	16.0 ± 7.6	375 ± 224	1.5 ± 0.6
Median												
Male	2,358	67.0	1,565	13.0	325	35.0	814	27.5	636	18.0	413	1.30
Female	2,203	66.0	1,497	15.0	340	35.0	813	27.0	597	14.0	330	1.33
Reference range												
Male	1,147–3,727	47.1–79.4	701–2,517	7.0–22.0	109–802	23.0–47.0	313–1,361	14.3–40.3	228–1,243	7.6–37.4	140–1,103	0.68–2.73
Female	1,342–3,859	49.2–80.0	800–2,751	7.2–31.1	138–645	21.5–48.8	399–1,489	12.6–41.4	254–1,193	5.2–36.6	96–998	0.70–3.22

[a] Absolute counts are given in cells per microliter. For males, $n = 104$; for females, $n = 128$.
[b] Mean ± SD values were compared by analysis of variance; only NK cell percentages and absolute counts were significantly different ($P < 0.05$).

age. The means, standard deviations, medians, and reference ranges for males and females are tabulated in Table 3. Only NK cell percentages and absolute counts were significantly different ($P < 0.0001$). These differences are not clinically relevant as quantification of NK cell subsets does not play a significant role in clinical management.

Our study did not show any significant variation in lymphocyte subsets according to age except for $CD8^+$-cell counts, which decreased with age ($P < 0.0001$; data not shown). When the analysis was done according to racial group, there were significant differences among the racial groups in the following parameters: $CD3^+$-T-cell counts, $CD19^+$-B-cell counts, and $CD4^+$-T-helper-cell counts (Table 4). Indians seemed to have higher $CD3^+$, $CD4^+$, and $CD19^+$ counts than the Chinese or Malays, while lymphocyte subsets were comparable between the Malays and Chinese.

We then compared the results of the Chinese subset in our population with the results obtained from a study of Chinese in Hong Kong. Hong Kong and Singapore have very similar socioeconomic and environmental characteristics. Genetically, the Chinese from these two populations should be closely related. The two study populations also have similar age distributions. Interestingly, despite having similar lymphocyte subset percentages, the absolute $CD3^+$-, $CD19^+$-, and $CD4^+$-cell counts of the Singapore cohort are significantly higher than those of the Hong Kong cohort (Table 5).

DISCUSSION

In this study, we have managed to obtain reference ranges for lymphocyte subsets in a multiracial cohort that should be representative of our adult population. These reference ranges are applicable to patients between the ages of 16 and 65 years, which is also the clinically relevant population. These reference ranges do not apply to the pediatric population. As some studies have shown the effect of age on lymphocyte subsets, a separate study is necessary to determine reference ranges for this group of patients.

The results obtained from our population are again different from the results of other populations studied. This difference suggests that each population should have its own reference ranges for lymphocyte subsets. As a result, populations where HIV and AIDS are a major problem should study their own HIV-AIDS cohorts to see if traditional thresholds for CD4 used for the determination of treatment and prophylaxis and for AIDS definition are applicable to their populations as these ranges were defined in terms of Caucasian populations. Immunological progression markers for HIV-AIDS may have to be reestablished for different populations, and this process will require long-term prospective cohort studies aimed at describing the progression of HIV in each population.

The sources of variations between populations are less certain. Comparisons are hampered by differences in the methodologies of lymphocyte subset analysis. Results from external quality assurance surveys in the early 1990s identified a range of methodological features that could affect the results of flow cytometric immunophenotyping: blood sample characteristics and integrity, sample preparation (red cell removal techniques, staining, washing, and fixation), staining reagents and fluorochromes, flow cytometer setup and performance, data acqui-

TABLE 4. Comparison of absolute lymphocyte subpopulation counts between different races in the study cohort

Cell type	Mean absolute count ± SD by race (cells/μl)			
	Chinese (n = 184)	Malay (n = 22)	Indian (n = 17)	Other (n = 9)
CD3 T cells[a]	1,547 ± 453	1,599 ± 518	1,881 ± 407	1,908 ± 458
CD4 T cells[a]	812 ± 255	856 ± 308	958 ± 296	1,096 ± 184
CD8 T cells[b]	629 ± 235	661 ± 257	707 ± 221	738 ± 261
B cells[a]	330 ± 132	422 ± 137	514 ± 411	342 ± 139
NK cells[b]	425 ± 244	363 ± 212	358 ± 189	527 ± 260

[a] Significant difference ($P < 0.05$) as indicated by a two-tailed Student t test.
[b] Not significant.

sition and analysis, including gating strategies, and absolute cell count assessment (12, 13, 17, 29). A multicenter study in 1995 highlighted three major technical difficulties resulting in interlaboratory variability: variations in sample processing, suboptimal lymphocyte gating that uses light scattering (forward scatter and side scatter [SSC]) and a CD14-CD45 strategy, and variations in white cell count measurement (14). More recent surveys have reported marked improvement in interlaboratory variability with the adoption of guidelines (44), improvements in gating strategies (using CD45 and SSC, due to the availability of more fluorochromes), and the adoption of the lyse–no-wash instead of the Ficoll-Hypaque method for sample preparation (15). Other studies have also shown that the major source of interlaboratory variation in flow cytometric analysis of lymphocyte subsets is the variation in lymphocyte count produced by the automated blood analyzer when a dual-platform method is used (21, 27). This fact is further illustrated by the comparison between results for the Chinese in our population and those for the Hong Kong Chinese. Despite showing similar percentages of lymphocyte subsets, the populations have different absolute values for $CD3^+$, $CD4^+$, and $CD19^+$ cells. This source of variation can be eliminated by the single-platform methodology (2) that is adopted in this study. Furthermore, most of the other studies trying to establish reference ranges for lymphocyte subsets adopted older gating strategies and sample preparation methods, making direct comparison difficult. As far as we know, ours is the first study that uses the single-platform method and the latest guidelines to establish reference ranges for lymphocyte subsets in an Asian population. Recently, a guideline on T-cell subset enumeration by single-platform flow cytometry was published by the Centers for Disease Control and Prevention (23). We suggest that future studies utilize this method and adopt recommendations from the latest guidelines to improve interlaboratory comparability.

Environmental factors like prevailing mycobacterium infection, hepatitis, helminthic infestation, and poor nutrition have been suggested as possible causes of the differences between populations in lymphocyte subsets. As a result, whether there are true racial and genetic differences is hard to ascertain. Our study is unique in that it looks at the interracial differences among a cohort that lives in one environment. Our study showed $CD3^+$-T-cell, $CD4^+$-T-helper-cell, and $CD19^+$-B-cell counts that were significantly higher in Indians than in Chinese and Malays. This trend is similar to that reported in a previous study, suggesting that it is probably true (8). Very little data

TABLE 5. Comparison between the Chinese cohorts of the Singapore study and of the Hong Kong study[a]

Cohort	Lymphocyte absolute count	Value for cell type:[b]										CD4/CD8 ratio
		CD3		CD19		CD4[c]		CD8		NK		
		% Positive	Absolute count	% Positive	Absolute count	% Positive	Absolute count	% Positive	Absolute count	% Positive	Absolute count	
Singapore (n = 184)	2,331 ± 665	67 ± 7.4	1,547 ± 453	15 ± 5	330 ± 132	35 ± 6	812 ± 255	27 ± 7	629 ± 235	18 ± 7	425 ± 244	1.0 ± 0.6
Hong Kong (n = 208)	1,981 ± 526[b]	69 ± 7.7[c]	1,370 ± 400[b]	11 ± 4[c]	221 ± 108[b]	36 ± 7.5	725 ± 256[b]	30 ± 7[c]	589 ± 205[c]	20 ± 8[c]	394 ± 194[c]	1.0 ± 0.4[c]

[a] Hong Kong study by Kam et al. (19). All values are means ± SD.
[b] Significant difference as indicated by a two-tailed Student t test ($P < 0.05$).
[c] Not significant.

exist for the Indian population. The number of Indians in our study is quite small, and a larger number of volunteers should be collected to confirm this finding.

Our study showed a significantly lower NK cell percentage and count in women than in men. This finding is consistent with findings of other studies (22, 32). However, we did not find any gender differences in $CD4^+$-T-helper-cell counts as has been reported in some other studies (3, 22, 32, 33, 40). Differences in immune cell numbers between genders may be secondary to the differential influences of sex hormones shown in murine studies (24). The mechanisms involved may include the modulation of thymic involution by sex hormones (16, 28) or the effect of binding to cell receptors for the sex steroid present on T cells (37).

Our study showed that the number of $CD8^+$ T cells increases with age and, as a result, the CD4/CD8 ratio decreases, similar to the findings of a previous Saudi study (35). The impact of age on lymphocyte subsets is not well established, with different studies reporting different results. Some studies showed that the number of $CD4^+$ cells increased while the number of $CD8^+$ cells decreased with age (9, 39), while others showed the numbers of both $CD4^+$ and $CD8^+$ cells increasing with age (45). The inconsistency arises because most studies looked at patients of different age ranges. Most of our cohort is between 20 and 49 years of age, so it would be difficult to draw any definite conclusion about the effect of age on lymphocyte subsets from our study. To answer this question, studies would need to include patients of a wide range of ages and with each age well represented. One such study including patients from birth onwards showed that the absolute lymphocyte count falls with age but that the percentage of $CD4^+$ and $CD8^+$ cells increases with age, thereby maintaining a relatively constant level of absolute $CD4^+$- and $CD8^+$-cell counts (10). Alternatively, a longitudinal study monitoring a cohort of healthy volunteers as they grow older would also provide more definitive results.

In conclusion, we have established reference ranges for the lymphocyte subsets in a multiracial Asian population by using four-color, single-platform flow cytometry and following the latest guidelines (lyse–no-wash sample preparation and CD45 and SSC lymphocyte gating). Our study suggests that there may be a true genetic difference in lymphocyte subsets and further confirms the gender difference in NK cell counts. The impact of age on the lymphocyte subset is less certain.

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