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 fluids 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.

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

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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-phycocerythrin–CD45-peridinin chlorophyll protein–CD4–allophycocyanin and CD3–fluorescein isothiocyanate–CD16 plus CD56–phycocerythrin–CD45-peridinin chlorophyll protein–CD19–allophycocyanin. The stained blood sample was lysed with a diluted lyzing 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 as 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 1. Age distribution according to gender

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

### TABLE 2. Lymphocyte subset percentages and absolute-number reference ranges of study population

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean</th>
<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+</td>
<td>29.2%</td>
<td>22.5–35.9%</td>
</tr>
<tr>
<td>CD4+</td>
<td>40.0%</td>
<td>30.0–50.0%</td>
</tr>
<tr>
<td>CD8+</td>
<td>14.0%</td>
<td>10.0–18.0%</td>
</tr>
<tr>
<td>NK</td>
<td>7.0%</td>
<td>5.0–9.0%</td>
</tr>
<tr>
<td>CD4/CD8</td>
<td>1.12</td>
<td>0.80–1.60</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Lymphocyte subset</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>66.0 ± 7.5</td>
<td>65.0 ± 8.0</td>
</tr>
<tr>
<td>CD4</td>
<td>23.0 ± 6.7</td>
<td>23.0 ± 7.2</td>
</tr>
<tr>
<td>CD8</td>
<td>11.0 ± 6.6</td>
<td>12.0 ± 7.0</td>
</tr>
<tr>
<td>CD3/CD8 ratio</td>
<td>2.97 ± 0.23</td>
<td>2.91 ± 0.27</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>3.00 ± 0.26</td>
<td>3.02 ± 0.23</td>
</tr>
<tr>
<td>CD3/CD4 ratio</td>
<td>2.21 ± 0.19</td>
<td>2.23 ± 0.20</td>
</tr>
<tr>
<td>CD3/CD8/CD4 ratio</td>
<td>3.03 ± 0.24</td>
<td>3.05 ± 0.22</td>
</tr>
<tr>
<td>CD3+ T-cell counts</td>
<td>1.6 ± 0.2</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>CD4+ T-cell counts</td>
<td>1.6 ± 0.2</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>CD19+ B-cell counts</td>
<td>1.6 ± 0.2</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>CD19- B-cell counts</td>
<td>1.6 ± 0.2</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>CD8+ T-cell counts</td>
<td>1.6 ± 0.2</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>CD8- T-cell counts</td>
<td>1.6 ± 0.2</td>
<td>1.6 ± 0.2</td>
</tr>
</tbody>
</table>

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-
showed CD3 among a cohort that lives in one environment. Our study is unique in that it looks at the interracial differences are true racial and genetic differences is hard to ascertain. Our populations in lymphocyte subsets. As a result, whether there been suggested as possible causes of the differences between nation, hepatitis, helminthic infestation, and poor nutrition have mendations from the latest guidelines to improve interlabora
gest that future studies utilize this method and adopt recom-
meration by single-platform
Asian population. Recently, a guideline on T-cell subset enu-
ting strategies and sample preparation methods, making direct
Furthermore, most of the other studies trying to establish
single-platform methodology (2) that is adopted in this study.
1995 highlighted three major technical difficulties resulting
sition and analysis, including gating strategies, and absolute
cell count assessment (12, 13, 17, 29). A multicenter study in

sion from the latest guidelines to improve interlabora-
tory variability with the adoption of guidelines (44), im-
provements 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 popu-
lations 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 enu-
neration by single-platform flow cytometry was published by
the Centers for Disease Control and Prevention (23). We sug-
ject that future studies utilize this method and adopt recom-
mandations from the latest guidelines to improve interlabora-
tory comparability.

Environmental factors like prevailing mycobacterium infec-
tion, 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>
<thead>
<tr>
<th>Cell type</th>
<th>Mean absolute count ± SD by race (cells/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chinese (n = 184)</td>
</tr>
<tr>
<td></td>
<td>1,547 ± 453</td>
</tr>
<tr>
<td>CD3 T cells</td>
<td>812 ± 235</td>
</tr>
<tr>
<td>CD8 T cells</td>
<td>629 ± 235</td>
</tr>
<tr>
<td>B cells</td>
<td>330 ± 132</td>
</tr>
<tr>
<td>NK cells</td>
<td>425 ± 244</td>
</tr>
</tbody>
</table>

* Significant difference (P < 0.05) as indicated by a two-tailed Student t test.

b Not significant.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Lymphocyte count</th>
<th>CD3⁺</th>
<th>CD4⁺</th>
<th>CD8⁺NK⁺</th>
<th>CD19⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absolute</td>
<td>Positive</td>
<td>%</td>
<td>Positive</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td>Value for cell type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chinese</td>
<td>208</td>
<td>1,981</td>
<td>665</td>
<td>67.2</td>
<td>15</td>
</tr>
<tr>
<td>Malay</td>
<td>212</td>
<td>358</td>
<td>235</td>
<td>26.1</td>
<td>41</td>
</tr>
<tr>
<td>Indian</td>
<td>221</td>
<td>738</td>
<td>257</td>
<td>11.9</td>
<td>66</td>
</tr>
<tr>
<td>Other</td>
<td>205</td>
<td>589</td>
<td>221</td>
<td>11.9</td>
<td>66</td>
</tr>
</tbody>
</table>

* Significant difference as indicated by a two-tailed Student t test (P < 0.05).
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, but that the percentage of CD4+ cells increases with age and, as a result, the CD4/CD8 ratio decreases, and any gender differences in CD4+ lymphopoeisis. Pediatr. Clin. N. Am. 47:1291–1310.


