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ORIGINAL ARTICLE

# Reference Values for Peripheral Blood Lymphocyte Subsets in a Healthy Korean Population

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Flow cytometric immunophenotyping of peripheral blood lymphocyte subsets is a powerful tool for evaluating cellular immunity and monitoring immune-mediated diseases. The numbers and proportions of blood lymphocyte subsets are influenced by factors such as gender, age, ethnicity, and lifestyle. This study aimed to establish reference ranges for peripheral blood lymphocyte subsets in a healthy Korean population. Blood samples from 294 healthy adults were collected. Lymphocyte subsets were analyzed using a single-platform method with a flow cytometer; white blood cells and lymphocytes were analyzed using an automated hematology analyzer. The mean value of the white blood cell count was 5,665 cells/$\mu$l, and the mean values of the subtype counts (percentages) were as follows: lymphocytes, 1,928 cells/$\mu$l (35.08%); $CD3^+$ cells, 1,305 cells/$\mu$l (67.53%); $CD3^+CD4^+$ cells, 787 cells/$\mu$l (40.55%); $CD3^+CD8^+$ cells, 479 cells/$\mu$l (25.23%); $CD3^-CD19^+$ cells, 203 cells/$\mu$l (10.43%); and $CD3^-CD56^+$ cells, 300 cells/$\mu$l (15.63%). Additionally, the $CD4^+/CD8^+$ ratio was 1.81. In this study, gender and age significantly influenced blood lymphocyte subsets. Our results demonstrate that, as with other populations, a healthy Korean population has its own, region-specific, lymphocyte subset reference ranges.
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Keywords: Flow cytometry, Immunophenotyping, Lymphocytes

## INTRODUCTION

Circulating lymphocytes play key roles in maintaining immune homeostasis against invading pathogens and damaged host-derived cells that might otherwise destroy the immune balance. Many immune-related disorders, such as autoimmune diseases, immunodeficiency syndromes, allergies, transplantation rejection, and leukemia, are involved in phenotypic alterations of lymphocyte subsets. For instance, autoimmune lymphoproliferative-syndrome (ALPS) is marked by an increase in the number of alpha/beta receptor-bearing $CD4^-CD8^-$, double-negative T-cells (1); human immunodeficiency virus (HIV) infection is associated with a decline in $CD4^+$ T-cells (2); and acute myeloid lymphoma (AML) is characterized by decreased expression of the CD45 antigen (3). Therefore, an examination of the phenotypic changes in blood lymphocyte subsets is useful for understanding the onset and progression of diseases and determining optimal treatment modalities.

Flow cytometric analysis is a precise and convenient method for studying immunophenotypic profiles in various cell types and has been widely used for the clinical diagnosis and management of immune diseases associated with phenotypic and functional perturbations of lymphocyte subsets (4,5). Thus, determining reliable reference values for the lymphocyte subsets in normal populations is essential, and many regional works have established local reference values, which are expressed as absolute counts and percentages of cells.

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Abbreviations: ALPS, autoimmune lymphoproliferative-syndrom; AML, acute myeloid lympoma; WBC, white blood cells; APC, allophycocyanin

However, such regional data show variations due to circadian fluctuations in blood lymphocytes (6) and the influence of gender, age, ethnicity, and lifestyle differences (4,7). These data indicate that different regional populations should have their own defined reference values for blood lymphocyte subsets. This study aimed to determine the reference ranges for blood lymphocyte subsets in healthy Korean adults and assess the influence of gender and age on lymphocyte subsets.

## MATERIALS AND METHODS

### Study population

Healthy individuals who visited the Seoul Songdo Colorectal Hospital for regular medical checkups between April and September 2013 were randomly recruited. All participants were examined and a questionnaire was administered to confirm their condition and health history. Only participants with serious medical problems, such as HIV infection, autoimmune disease, allergies, and cancer; using medications or dietary supplements; or pregnant at the time of the study, were excluded.

### Blood collection

Blood samples were managed at room temperature and processed within 1 h on the day of collection. Whole blood was collected aseptically into potassium ethylenediaminetetraacetic acid-containing tubes (Vacuette®, Greiner bio-one, Kremsmünster, Austria) for complete blood counts and differential counts using an automated hematology analyzer (Sysmex XE-2100, Kobe, Japan), and for immunophenotyping by flow cytometry. The parameters for analysis were the numbers of white blood cells (WBC), and the percentages and numbers of lymphocytes, $CD3^+$ cells, $CD3^+CD4^+$ cells, $CD3^+CD8^+$ cells, $CD3^-CD19^+$ cells, and $CD3^-CD56^+$ cells; the $CD4^+/CD8^+$ ratio was also determined.

### Flow cytometric analysis

Aliquots (50 $\mu$l) of whole blood were added to 12×75-mm capped polypropylene test tubes containing premixed monoclonal antibodies (mAbs). The mAbs against CD3, CD4, CD8, CD56 and CD19 (BD Pharmingen™, San Diego, CA, USA), and the mAb against CD45 (eBioscience, San Diego, CA, USA) used in this study were purchased at the same time and were from the same lot number. The optimal working concentration for each antibody was determined by serial dilution of the antibody in a preliminary titration experiment. Two sets of antibody cocktails were prepared using the determined amount of each antibody. Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD3 mAb, phycoerythrin (PE)-conjugated mouse anti-human CD8 mAb, PE cyanin5-conjugated mouse anti-human CD4 mAb, and allophycocyanin (APC)-conjugated mouse anti-human CD45 mAb were mixed in one test tube, and FITC-conjugated mouse anti-human CD3 mAb, PE-conjugated mouse anti-human CD56 mAb, PE cyanin5-conjugated mouse anti-human CD19 mAb, and APC-conjugated mouse anti-human CD45 mAb were mixed in the other test tube. Blood, labeled with each set of antibodies, was incubated in a dark room for 20 min at room temperature and the red blood cells were lysed by adding 440 $\mu$l of FACS lysing solution (BD Biosciences, San Jose, CA, USA) for 15 min. Then, 50 $\mu$l of CountBright™ absolute counting beads (Molecular Probes, Carlsbad, CA, USA) was added to each cell-staining tube and immediately assessed using a flow cytometer (BD FACSCalibur, San Jose, CA, USA); the results were analyzed using CellQuest™ pro software (BD, San Jose, CA, USA).

### Quality control

Daily calibration of the flow cytometer was performed using the manufacturer's software program (FACSComp software, BD) and materials (Calibrated beads, BD), and the intensity of the antibody fluorescence was monitored every week to ensure consistent results.

### Statistical analysis

Data were analyzed using the SPSS statistical package program, version 18 (IBM, Armonk, NY, USA). The mean and standard deviation values were calculated for all parameters. A Kolmogorov-Smirnov test and a Shapiro-Wilks test were used to check the Gaussian distribution of all variables. Because all variables had non-Gaussian distributions, the reference ranges for all parameters were determined using the 95th percentile of the area under the distribution curve of the variable. An Analysis of covariance (ANCOVA) test was used to compare variables between men and women, and a Kruskal-Wallis test with Spearman's correlation coefficients was used to check for significant differences between age groups. p-values $<0.05$ were considered statistically significant.

### Ethic statements

This study was approved by the institutional review board of Seoul Song Do Colorectal Hospital (IRB Number 2012-006). Informed consent was waived by the board because blood sampling was performed for clinical examinations but verbal consent was obtained from all participants at the time of blood draw.

## RESULTS

Among the recruited volunteers, a total of 294 normal, healthy donors (mean age, 47±14 years old) met our criteria and had blood samples processed for the study. Absolute counts and/or percentages of the indicated cell populations are presented as mean values with standard deviations, medians, and 95% confidence intervals; reference ranges were extracted from the 2.5 to 97.5 percentile (Table I).

### Influence of gender

The study population consisted of 139 men and 155 women (mean age; 49±13 and 45±14 years old, respectively). A comparison of the parameters between these 2 groups is presented in Table II. Overall, statistically significant differences in several parameters were observed between the groups. The percentages of $CD3^+$ cells and $CD3^+CD4^+$ cells were lower in men than in women ($p<0.05$, in both cases), but the absolute count and percentage of $CD3^-CD56^+$ cells were higher in men than in women ($p<0.001$, in both cases). However, no significant differences between men and women were observed with respect to the other subtypes.

### Influence of age

In order to examine the changes in the cell subtypes according to age, the healthy donors, 21~80-years-old, were classified into 5 groups. Statistically significant differences in absolute counts and percentages of $CD3^+$ cells, $CD3^+CD8^+$ cells, and $CD3^-CD56^+$ cells were observed between the groups ($p<0.05$). Likewise, the percentages of lymphocytes and the $CD4^+/CD8^+$ ratio were also significantly different between the groups ($p<0.01$) (Table III). These observations were

**Table I.** Reference ranges of absolute counts and percentages of lymphocyte subsets in a healthy Korean population

| Parameter | Mean±SD | Median | Confidence interval[a] | Reference range[b] |
|---|---|---|---|---|
| WBC | | | | |
| Cells/μl | 5,665±1,528 | 5,500 | 5,490~5,841 | 3,200~9,100 |
| Lymphocytes | | | | |
| Cells/μl | 1,928±508 | 1,887 | 1,870~1,987 | 1,178~3,262 |
| % | 35.08±8.40 | 34.75 | 34.12~36.05 | 17.08~50.90 |
| $CD3^+$ | | | | |
| Cells/μl | 1,305±403 | 1,243 | 1,259~1,351 | 708~2,294 |
| % | 67.53±9.64 | 68.34 | 66.42~68.63 | 45.31~82.81 |
| $CD3^+CD4^+$ | | | | |
| Cells/μl | 787±290 | 714 | 753~820 | 394~1,574 |
| % | 40.55±8.54 | 40.00 | 39.57~41.53 | 25.94~59.73 |
| $CD3^+CD8^+$ | | | | |
| Cells/μl | 479±179 | 460 | 458~499 | 188~830 |
| % | 25.23±7.49 | 24.92 | 24.37~26.09 | 11.38~40.51 |
| $CD3^-CD19^+$ | | | | |
| Cells/μl | 203±100 | 182 | 191~214 | 57~461 |
| % | 10.43±3.75 | 10.05 | 10.00~10.86 | 3.90~19.70 |
| $CD3^-CD56^+$ | | | | |
| Cells/μl | 300±162 | 260 | 282~319 | 91~682 |
| % | 15.63±7.25 | 13.93 | 14.80~16.47 | 5.11~33.78 |
| $CD4^+/CD8^+$ | | | | |
| Ratio | 1.81±0.89 | 1.61 | 1.71~1.92 | 0.77~4.42 |

[a]95% confidence interval for sample mean. [b]2.5th~97.5th percentile of reference range

**Table II.** Comparison of absolute counts and percentages of white blood cells and lymphocyte subsets in 139 males and 155 females

| Parameter | Mean±SD | | Reference range[a] | | p-value[b] |
|---|---|---|---|---|---|
| | Male | Female | Male | Female | |
| Age | | | | | |
| Years old | 49±13 | 45±14 | | | 0.010[c] |
| WBC | | | | | |
| Cells/μl | 5,706±1,284 | 5,628±1,722 | 3,700~8,850 | 3,080~9,780 | 0.581 |
| Lymphocytes | | | | | |
| Cells/μl | 1,966±466 | 1,895±542 | 1,152~3,122 | 1,165~3,327 | 0.279 |
| % | 35.12±7.99 | 35.05±8.78 | 20.65~47.90 | 16.12~52.29 | 0.840 |
| $CD3^+$ | | | | | |
| Cells/μl | 1,295±395 | 1,314±411 | 704~2,301 | 692~2,302 | 0.957 |
| % | 65.56±10.23 | 69.28±8.74 | 39.26~82.78 | 46.14~83.05 | 0.012 |
| $CD3^+CD4^+$ | | | | | |
| Cells/μl | 763±272 | 808±305 | 396~1,521 | 394~1,661 | 0.132 |
| % | 38.69±8.77 | 42.22±7.99 | 24.68~61.92 | 26.23~59.51 | <0.001 |
| $CD3^+CD8^+$ | | | | | |
| Cells/μl | 490±197 | 469±161 | 180~879 | 196~814 | 0.057 |
| % | 25.13±7.89 | 25.33±7.13 | 10.58~41.77 | 11.39~40.81 | 0.316 |
| $CD3^-CD19^+$ | | | | | |
| Cells/μl | 210±101 | 196±100 | 47~450 | 61~464 | 0.285 |
| % | 10.57±3.80 | 10.30±3.70 | 3.00~19.87 | 3.80~19.91 | 0.628 |
| $CD3^-CD56^+$ | | | | | |
| Cells/μl | 339±172 | 265±145 | 120~762 | 83~646 | <0.001 |
| % | 17.56±7.93 | 13.91±6.12 | 5.66~35.84 | 4.752~9.01 | <0.001 |
| $CD4^+/CD8^+$ | | | | | |
| Ratio | 1.77±1.00 | 1.85±0.78 | 0.77~4.79 | 0.77~3.80 | 0.010 |

[a]2.5th~97.5th percentile of reference range. [b]Analysis of covariance (ANCOVA) test. [c]Student's *t* test

further analyzed by calculating Spearman's correlation coefficients. A trend of decreasing absolute counts and percentages of $CD3^+$ cells ($r=-0.162$, $p=0.005$ and $r=-0.383$, $p<0.001$) and $CD3^+CD8^+$ cells ($r=-0.316$ and $r=-0.467$; $p<0.001$, in both cases) with increasing age were observed. On the other hand, the percentage of lymphocytes ($r=0.128$, $p=0.029$) and the absolute count and percentage of $CD3^-CD56^+$ cells ($r=0.378$ and $r=0.371$; $p<0.001$, in both cases) showed a tendency to increase with increasing age. Consequently, the $CD4^+/CD8^+$ ratio ($r=0.372$, $p<0.001$) also showed a gradual increase with increasing age, owing to a significant decrease in the percentage of $CD3^+CD8^+$ cells. However, no significant differences or correlations, according to age, were detected among the 5 groups of the other lymphocyte subtypes (Table IV).

## DISCUSSION

In this study, we investigated the reference ranges for peripheral blood lymphocyte subsets in a healthy Korean population. The results of this large-scale study enable the use of region-specific data when examining and diagnosing Korean adults with clinical disorders characterized by alterations in blood lymphocyte subsets. The availability of these Korea-specific data reduces the need to rely on Western or Chinese data.

Similar to previously presented findings, our results demonstrated differences from data obtained in other regions and countries. Such differences could be due to the influence of inherent biological parameters (e.g., gender, age, and ethnicity) and lifestyle factors (e.g., alcohol use, smoking, diet, exercise, and stress levels) on blood lymphocyte populations (8,9). In fact, previous reports, performed in countries with

**Table III.** Absolute counts and percentages of lymphocyte subsets as per age groups

| Parameter | Mean±SD | | | | | p-value[a] |
|---|---|---|---|---|---|---|
| | 21~30 (n=38) | 31~40 (n=67) | 41~50 (n=64) | 51~60 (n=69) | 61~80 (n=49) | |
| WBC | | | | | | |
| Cells/μl | 5,953±1,650 | 5,759±1,603 | 5,609±1,442 | 5,323±1,210 | 5,865±1,779 | 0.229 |
| Lymphocytes | | | | | | |
| Cells/μl | 1,806±537 | 1,914±410 | 2,021±519 | 1,896±412 | 1,972±679 | 0.164 |
| % | 31.29±7.75 | 34.28±6.52 | 36.87±9.55 | 36.52±7.36 | 34.83±10.00 | 0.007 |
| $CD3^+$ | | | | | | |
| Cells/μl | 1,316±389 | 1,356±331 | 1,376±411 | 1,246±366 | 1,220±514 | 0.015 |
| % | 73.07±6.73 | 70.77±6.91 | 68.09±9.57 | 65.22±9.97 | 61.40±10.28 | <0.001 |
| $CD3^+CD4^+$ | | | | | | |
| Cells/μl | 727±283 | 750±206 | 829±303 | 801±287 | 809±371 | 0.251 |
| % | 39.90±7.01 | 39.30±7.05 | 41.11±10.17 | 41.66±9.43 | 40.48±7.92 | 0.645 |
| $CD3^+CD8^+$ | | | | | | |
| Cells/μl | 543±149 | 529±152 | 509±216 | 420±167 | 406±155 | <0.001 |
| % | 30.27±6.20 | 28.63±5.86 | 25.10±7.55 | 22.13±7.36 | 21.26±6.27 | <0.001 |
| $CD3^-CD19^+$ | | | | | | |
| Cells/μl | 186±120 | 204±76 | 200±92 | 207±94 | 211±129 | 0.334 |
| % | 9.94±3.58 | 10.74±3.52 | 9.74±3.37 | 10.82±3.56 | 10.71±4.72 | 0.565 |
| $CD3^-CD56^+$ | | | | | | |
| Cells/μl | 208±94 | 255±132 | 319±209 | 329±147 | 369±147 | <0.001 |
| % | 11.45±3.80 | 13.27±5.71 | 15.59±8.42 | 17.68±7.57 | 19.26±6.53 | <0.001 |
| $CD4^+/CD8^+$ | | | | | | |
| Ratio | 1.41±0.54 | 1.45±0.46 | 1.85±0.91 | 2.15±1.09 | 2.12±0.95 | <0.001 |

[a]Kruskal-Wallis test

huge populations and significant socio-demographic diversity, such as China, India, and Brazil, showed region-specific differences in lymphocyte subsets (10-12). These observations suggest that each population should have its own reference ranges for blood lymphocytes and that such reference values should be regularly updated as socio-demographic factors change. The most parameters in our study are relatively different with those from other populations studied. For example, the median number of NK cells in our study is higher than that studied in Switzerland and Turkey but lower than that studied in China and Tanzania (13-16).

The influences of gender and age on the lymphocyte subsets were also analyzed. First, we observed significant gender differences in some lymphocyte subsets. These differences are thought to be mainly caused by the effect of sex hormones (17,18). However, the socio-demographic and lifestyle factors characterizing each population also seem to contribute to and influence such gender differences in lymphocyte subsets because the pattern of differences between men and women are quite distinct, depending on the population studied. For instance, in our study, the absolute count and percentage of $CD3^-CD56^+$ cells were higher among men than among women and a similar observation has been addressed in some (9,11,19), but not all, (16,20,21) regional reports.

We also found age-associated changes in some lymphocyte subsets. In particular, with advancing age, we observed tendencies towards decreased absolute count and percentage of $CD3^+CD8^+$ cells and towards increasing absolute count and percentage of $CD3^-CD56^+$ cells; these findings are consistent with those of other reports (15,19). In general, aging affects the potential activity of hematopoietic stem cells, the involution of the thymus, and the decline in T lymphocytes (22). Similarly, aging influences NK-cell activity and phenotypic changes; although NK-cell activity tends to decline, the overall function of NK-cells in healthy individuals does not change owing to an increase in the number of NK-cells (23,24). Few reports have addressed the correlations between

**Table IV.** Correlations of age with absolute counts and percentages of lymphocyte subsets

| Parameter | Correlation coefficient (r) | p-value |
|---|---|---|
| WBC | | |
| Cells/$\mu$l | −0.061 | 0.301 |
| Lymphocytes | | |
| Cells/$\mu$l | 0.012 | 0.842 |
| % | 0.128 | 0.029 |
| $CD3^+$ | | |
| Cells/$\mu$l | −0.162 | 0.005 |
| % | −0.383 | <0.001 |
| $CD3^+CD4^+$ | | |
| Cells/$\mu$l | 0.056 | 0.340 |
| % | 0.068 | 0.240 |
| $CD3^+CD8^+$ | | |
| Cells/$\mu$l | −0.316 | <0.001 |
| % | −0.467 | <0.001 |
| $CD3^-CD19^+$ | | |
| Cells/$\mu$l | 0.062 | 0.288 |
| % | | |
| $CD3^-CD56^+$ | | |
| Cells/$\mu$l | 0.378 | <0.001 |
| % | 0.371 | <0.001 |
| $CD4^+/CD8^+$ | | |
| Ratio | 0.372 | <0.001 |

$CD3^+CD8^+$ and $CD3^-CD56^+$ cell counts and age, but adaptive immunity is believed to functionally decline with advancing age (25). Thus, the immune system may be postulated to have evolved in such a way as to be programmed to balance declining numbers of cytotoxic T-cells (involved in adaptive immunity) with increasing numbers of NK-cells (innate immunity).

This study has a limitation in terms of the analysis. Our data were analyzed against 2 biological parameters−gender and age−without considering other socio-demographic and lifestyle factors. This was the result of a lack of information on the other factors derived from the questionnaires completed by the subjects. We hope to include the analysis of such complicated factors in an updated report.

In conclusion, this study established a profile for peripheral blood lymphocyte subsets in a healthy Korean population. These data may be used as standard reference values for the Korean population; they support the idea that each population should have its own region-specific reference values that reflect various socio-demographic factors. Finally, our data may be used worldwide as reference data for comparative analyses of blood lymphocyte subsets.

## CONFLICTS OF INTEREST

The authors have no financial conflict of interest.

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