Original Article

Establishment of the reference intervals of lymphocyte subsets for healthy Chinese Han adults and its influencing factors

Kangli Xu[1,2#^], Linzi Miao[3#], Weiye Chen[4#], Hui Wu[1,2], Yan Gong[3], Xiaoxin Tu[4], Wei Guo[1,2], Baishen Pan[1,2], Chenxue Qu[3], Xinzhong Wu[5], Beili Wang[1,2^]

[1]Department of Laboratory Medicine, Zhongshan Hospital, Fudan University, Shanghai, China; [2]Branch of National Clinical Research Center for Laboratory Medicine, Shanghai, China; [3]Department of Clinical Laboratory, Peking University First Hospital, Beijing, China; [4]Department of Laboratory Medicine, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, China; [5]Department of Transfusion Medicine, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, China

Contributions: (I) Conception and design: C Qu, X Wu, B Wang; (II) Administrative support: W Guo, B Pan; (III) Provision of study materials or patients: H Wu, Y Gong, X Tu; (IV) Collection and assembly of data: L Miao, W Chen, H Wu; (V) Data analysis and interpretation: K Xu, H Wu, Y Gong, X Tu; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work and are co-first authors.

Correspondence to: Beili Wang. Department of Laboratory Medicine, Zhongshan Hospital, Fudan University, 180 Feng Lin Road, Shanghai 200032, China. Email: wang.beili1@zs-hospital.sh.cn; Chenxue Qu. Department of Clinical Laboratory, Peking University First Hospital, Peking University First Hospital, Beijing 100034, China. Email: qucx2012@163.com; Xinzhong Wu. Department of Transfusion Medicine, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou 510120, China. Email: wuxinzhong5054@163.com.

Background: Cellular immune monitoring is becoming more critical in the clinic, but its application has not yet become sufficiently widespread. One reason may be the different reference intervals among clinical laboratories due to several factors. Percentage and number of lymphocyte subsets are standard indicators of cellular immune detection. The present study aimed to establish standardized reference intervals of lymphocyte subsets in the healthy Chinese Han adult population and examine such influencing factors as age, gender, region, and measurement instruments.

Methods: A total of 496 healthy Chinese Han people aged 18–59 years from 3 China Mainland regions (north, east, and south) were enrolled. The sample of each center was simultaneously examined by three flow cytometers (FACSCanto^{TM}II, FACSLyric^{TM}, and FACSCalibur^{TM}). A single-platform flow cytometry-based absolute count technique was used to quantify the percentage and number of each lymphocyte subset. The flow cytometry results were analyzed by variance analysis and Z test to determine the influence of age, gender, and instruments on lymphocyte subsets.

Results: Multi-center, age-specific, and gender-specific reference intervals of healthy Chinese Han adults' lymphocyte subsets were established. There was no statistical difference in the results from the three flow cytometers. Gender affected the results of $CD4^+$ (%) and the absolute count of $CD3^-CD16^+CD56^+$, where $CD4^+$ (%) was higher in women, and the absolute count of $CD3^-CD16^+CD56^+$ was higher in men. Age mainly affected the $CD4^+/CD8^+$ ratio, which was statistically higher in groups aged over 40 years; the percentage and number of $CD3^-CD19^+$ were more elevated in age groups below 30 years; however, the difference was not statistically significant.

Conclusions: This study established the reference intervals of lymphocyte subsets for healthy Chinese Han adult populations under the standardized methods. This study was the first nationwide study in China to use a flow cytometry-based single-platform method to establish the reference intervals of lymphocyte subsets of the healthy Chinese Han adult population. Gender and age were shown to influence the results of lymphocyte subsets.

^ ORCID: Kangli Xu, 0000-0003-1648-0443; Beili Wang, 0000-0001-5167-170X.

Keywords: Lymphocyte subsets; reference interval; flow cytometry; single-platform; cellular immunity

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Introduction

The maintenance of the normal immune function in the body depends not only on humoral immunity but also on cell-mediated immune responses. Various immune cells are involved in elaborate immune regulation, especially lymphocyte subsets, to produce a reasonable immune reaction. The number of lymphocytes and their subset ratios may change when an immune malfunction occurs in the states of allergic diseases, autoimmune diseases, immunodeficiency diseases, viral infections, and cancer treatments (1-5). Unfortunately, when physicians assess the results of various patients' examinations, they gravitate towards classical indicators, those from clinical chemistry or humoral immunity, rather than from cellular immunity. One of the most common detections in cellular immunity is lymphocyte subsets, including T lymphocytes ($CD3^+$), B lymphocytes ($CD3^-CD19^+$), helper/inducible T lymphocytes ($CD3^+CD4^+$), inhibitory/cytotoxic T lymphocytes ($CD3^+CD8^+$), and natural killer (NK) cells ($CD3^-CD16^+$ or $CD56^+$).

One reason cellular immune detections have not been given enough attention is their direct connection to the disease, and another reason could be the reference interval. For the former, fortunately, increasing research is being conducted. Once the peripheral blood white blood cell count and its classification are considered as traditional biomarkers in infectious diseases, in addition to C reactive protein (CRP), procalcitonin (PCT), and IL-6 (6). And the lymphocyte subsets detection has become one of the essential immune monitoring methods for patients with COVID-19 during this pandemic. The lymphocyte subsets provide clinical opportunities to understand the body's cellular immunity condition and evaluate its immune function or immunoreactivity, which plays a vital role in disease evaluation, prognosis evaluation, and efficacy assessment (7-12). Although most of the early studies are from China, the reference intervals of lymphocyte subsets are not homogenous (7,11). Because the reference intervals of lymphocyte subsets used in laboratories are mainly derived from reagent instructions, textbooks, and literature reports, they may be different. Only a few laboratories have established single-center reference intervals, which are not universally representative due to their potential differences in recruitment criteria and detection systems, including using different instruments and detecting methods. It is challenging to establish reference intervals of peripheral blood lymphocyte subsets due to many factors that affect the results, such as recruitment criteria, the patients' age and gender, region, and detection systems (13-23). However, it is still necessary to establish appropriate reference intervals for as vast applications as possible.

To establish reference intervals of lymphocyte subsets widely suitable for healthy Chinese Han adults, healthy participants through three centers distributed in different regions (Beijing from northern China, Shanghai from eastern China, Guangzhou from southern China) were recruited in this study. The percentage and absolute count of lymphocyte subsets in peripheral blood samples were simultaneously detected in each center's three flow cytometers via the single-platform detection method. Multi-center, age-specific, and gender-specific reference intervals of lymphocyte subsets were established, considering the influence of instruments on lymphocyte subset results. We present the following article in accordance with the MDAR reporting checklist (available at https://dx.doi.org/10.21037/atm-21-4031).

Methods

Study design and participants

The present study was conducted at three centers in mainland China: Zhongshan Hospital Affiliated to Fudan University (eastern China), Peking University First Hospital (northern China), and The Second Affiliated Hospital of Guangzhou University of Chinese Medicine (southern China). A total of 1,112 Chinese Han adults aged 18–59 from March 2019 to November 2019 were screened, and 496 were enrolled for analysis. The ethics committees of each center approved this study [ethics approval number: Zhongshan Hospital Affiliated to Fudan University, B2018-

115; Peking University First Hospital, (2018) Research No. (145); Guangdong Provincial Hospital of Traditional Chinese Medicine, BF2018-087-01], and all participants provided written informed consent. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013).

Inclusion criteria

The inclusion criteria were as follows: (I) participation in physical examination; (II) completion of questionnaire survey prompts health assessment.

Exclusion criteria

The exclusion criteria were as follows: (I) patients infected with hepatitis B virus (HBV), hepatitis C virus (HCV), AIDS (HIV), or syphilis; (II) infection with other acute and chronic infectious diseases; (III) autoimmune disorders, allergic diseases, or cancer; (IV) patients had taken drugs that affect the immune system within 3 months [including glucocorticoids, alkylating agents, antimetabolites, nucleotide reductase or tyrosine kinase inhibitors, botanicals, rapamycin target molecule inhibitors, biological drugs, monoclonal antibodies, and so on (17)]; (V) vaccination within 4 weeks; (VI) patients had received a blood transfusion within one year; (VII) patients had received blood donations within 6 months.

Study methods

Blood collection and sample processing

Approximately 2 mL of blood was collected from each fasting participant in ethylene diamine tetraacetic acid (EDTA) tubes (BD, Catalog No. 367856) and was transported at room temperature to the clinical laboratories of the 3 centers. Within 48 h after being collected, the anticoagulated whole blood sample was dyed and tested in the flow cytometer. To ensure that the number of lymphocytes was within a linear range, a white blood cell (WBC) differential count was performed before the sample was stained.

Flow cytometry

Flow cytometry tests were performed in the clinical laboratories of the 3 centers. In this study, 3 flow cytometers and their corresponding software were used, including FACSCalibur™ and Multiset™ software (BD), BD FACSCanto™II and FACSCanto™ software (BD), and BD FACSLyric™, and FACSuite™ software (BD). The antibody detection kit, Multitest™ IMK kit (BD, Catalog No. 340503), contains antibody Panel A [CD3-fluorescein isothiocyanate (FITC)/CD8-phycoerythrin (PE)/CD45-peridinin-chlorophyll protein (PerCP)/CD4-allophycocyanin (APC) antibodies] and antibody Panel B (CD3-FITC/CD16+56-PE/CD45-PerCP/CD19-APC antibodies). Multitest™ IMK kit and Trucount™ Absolute Counting Tubes (BD, Catalog No. 340334) were used to identify and quantify the percentage and number of lymphocyte subsets in erythrocyte-lysed whole blood samples.

To obtain the same test quality across the 3 centers, the flow cytometer's daily setup and automatic quality control were established before testing the selected samples. The same batch of quality control blood (BD Multi-Check Control, Catalog No. 349702; BD Multi-Check CD4 Low Ctrl, Catalog No. 349705) was used to perform quality control on the flow cytometers to meet the quality evaluation standards, including: (I) the results of quality control products were all in the fluctuation range of quality control; and (II) the results among laboratories were comparable. Meanwhile, to compare the flow cytometry results between different centers, the same batch of BD quality control blood was stained and measured 10 times following the laboratory standard operating procedures (SOPs) according to the instrument and reagent instructions. We took the mean (mean) ±2 standard deviation (SD) as the quality control range, the mean I was defined as the mean value of the measured value, SD = mean × line standard coefficient of variation (CV); when the positive percentage of the test index was ≥30%, the CV value was 8%; when the positive rate was less than 30%, the CV value was 15% [according to the "YY/T 0588-2017 Flow Cytometer Industry Standard implemented on 2018-12-1" (24)]. When 80% and above (≥80%) of the actual measured value fell within the quality control range, it was indicated that the quality control had passed.

The samples were detected according to the SOP for flow cytometry. Briefly, 20 μL antibody Panel A and 20 μL antibody Panel B reagents were added to Trucount A and Trucount B respectively; 50 μL anticoagulated whole blood was added to each tube by reverse aspiration method, they were gently mixed and incubated at room temperature (20–25 ℃) in the dark for 15 min, then (1×) lysing solution (diluted by BD FACS™ Lysing Solution 10X Concentrate, Catalog No. 349202) 450 μL was added to the 2 tubes

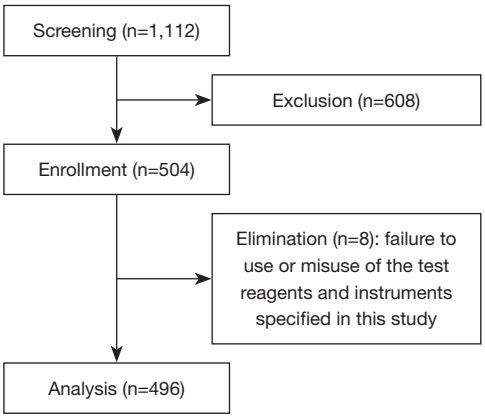

Figure 1 Collection, screening, elimination, and final enrollment of participants.

respectively, mixed well, incubated at room temperature for 15 min in the dark, and then tested with the flow cytometer. If not examined immediately, the stained sample was stored in the dark at room temperature and tested within 24 h. Flow cytometers were used to collect the cells, and the software was used to determine the percentage and number of each lymphocyte subset.

Gating Strategy. Tube A: SSC/CD45 is used to gate the lymphocyte population ($SSC^{low}CD45^{bright}$), SSC/APC is used to gate the Beads, SSC/CD3 is used to gate the $CD3^+$ T lymphocytes in the lymphocyte population, and then T lymphocytes are distinguished by $CD4^+CD8^-$ (helper/inducible T lymphocytes) cell group, $CD4^-CD8^+$ (inhibitory/cytotoxic T lymphocytes) cell group, $CD4^+CD8^+$ cell group, $CD4^-CD8^-$ cell group using CD4/CD8. Tube B: SSC/CD45 is used to gate the lymphocyte population ($SSC^{low}CD45^{bright}$), SSC/APC is used to gate the Beads, SSC/CD3 is used to gate the $CD3^-$ lymphocytes in the lymphocyte population, and then CD19/CD16&56 is used to distinguish $CD3^-$ lymphocytes to $CD19^+CD16\&56^-$ (B) Lymphocyte population, $CD19^-CD16\&56^+$ (NK) lymphocyte population.

Statistical analysis

Statistical analysis was carried out using SAS 9.4 software (SAS Institute Inc., Cary, NC, USA). According to the "WST 402-2012 Reference Intervals for Clinical Laboratory Test Items" implemented on 1 August 2013 (25), the outliers of the 3 flow cytometry test results: $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^-CD19^+$, $CD3^-CD16^+CD56^+$ absolute counts (pcs/μL) and percentage (%) and the measured values of $CD4^+/CD8^+$ ratio were arranged in ascending order to obtain adjacent measurements. The maximum value of the value difference was compared with 1/3 of the total distance (R) of all the indicator's measured values. When the results were greater than R/3, it was considered an outlier and subsequently eliminated. We compared the test results of the 3 flow cytometers: if the data was normally distributed, the analysis of variance (ANOVA) test with a completely random design was used, and the pairwise comparison between the groups used the least significant difference (LSD) test; if the data was non-normally distributed, the Kruskal-Wallis test was used for comparison between the groups, and Nemenyi test was used for pairwise comparison between groups. We used nested ANOVA to test the influence of the above factors on the value of lymphocyte subsets according to the hierarchical relationship of gender-age-region, and combined the scatter plot and Z test results to comprehensively consider whether to group to establish reference intervals for lymphatic subsets (17-19). We used P2.5–P97.5 to set the reference intervals of each lymphatic subset.

Results

Enrollment

This study screened 1,112 potential research participants, of which 504 were successfully enrolled, and 608 were excluded due to abnormalities in the questionnaire survey, physical examinations, and laboratory tests. Among the participants, 8 cases (1.6%) were excluded due to not using or misusing the test reagents and equipment specified, resulting in a final sample size of 496 cases (98.4%) meeting the protocol for analysis. The screening, enrollment, elimination, and final analysis of participants are displayed in *Figure 1*, and the demographic characteristics of the 496 participants are summarized in *Table 1*.

Quality control and comparison of the results in 3 flow cytometers

The quality control of 3 flow cytometers in each center was completed before starting the experiment, and all instruments passed the quality control (Table S1). The detection results of lymphocyte subsets appeared to have no statistical difference among the 3 devices except for the absolute count of $CD3^-CD16^+CD56^+$ cells and $CD3^+$ cells

Table 1 Demographic characteristics of the 496 enrolled participants

Age (years)	Region of center						Total
	Shanghai		Beijing		Guangzhou		
	Male	Female	Male	Female	Male	Female	
18–29	25	19	19	19	14	28	124
30–39	21	18	22	11	16	34	122
40–49	24	26	22	15	9	29	125
50–59	24	16	30	19	7	29	125
Total	94	79	93	64	46	120	496

(%). Further pairwise comparisons of the absolute count of $CD3^-CD16^+CD56^+$ cells and $CD3^+$ cells (%) showed no statistical difference. Therefore, the detection results of the 3 flow cytometers were averaged for subsequent analyses. Also, one data of $CD3^-CD19^+$ cells (%) in FACSCanto™II was eliminated as outliers before analysis, but the enrolled number was not changed.

Age, gender, and geographic distribution of lymphocyte subsets

Figure S1 shows the scatter plot of the age and gender distribution of each lymphocyte subset. It can be seen from the figure that women had higher $CD4^+$ cells (%) than men, while men's absolute counts of $CD3^-CD16^+CD56^+$ cells were higher than women's; the absolute counts of $CD3^-CD19^+$ cells, $CD3^-CD19^+$ cells (%), and $CD4^+/CD8^+$ ratios were age-specific. In detail, the absolute counts and percentages of $CD3^-CD19^+$ cells in men under 30 years old were higher than those in the over 30 years old group, and the $CD4^+/CD8^+$ ratio of people over 40 years old was higher than that of people under 40 years old. The results of the nested ANOVA showed that gender affected the lymphocyte subsets results except for the number of $CD3^+$ cells and $CD3^+CD8^+$ cells (%); the absolute count of $CD3^+$ cells, $CD3^+CD4^+$ cells (%), the percentage and number of $CD3^+CD8^+$ cells, and $CD4^+/CD8^+$ ratios were statistically different among different age groups; besides, the absolute counts of $CD3^-CD19^+$ cells in 3 regions had statistical differences (Table S2). The Z test results and difference analysis by gender are shown in Table S3: the absolute count of $CD3^-CD16^+CD56^+$ cells, the ZZ* difference of $CD3^-CD16^+CD56^+$ cells (%) and $CD3^+CD4^+$ cells (%) are greater than 0, which implies that the difference was statistically significant. In summary, there was a gender difference in the absolute counts of $CD3^-CD16^+CD56^+$ cells and $CD3^+CD4^+$ cells (%), while the $CD4^+/CD8^+$ ratio had an age difference; the above indicators need to be identified to establish reference intervals.

Reference intervals of lymphocyte subsets

Refer to *Table 2* for the reference intervals of lymphocyte subsets for healthy Chinese Han adults.

Discussion

Previous studies have shown that many laboratories have established reference intervals of lymphocyte subsets based on the region or the laboratory. These studies have used different detection platforms, detection methods, and enrollment criteria; moreover, most have been single-center studies, thereby not representative, which results in limitations. This study was the first multi-center study on the reference intervals of lymphocyte subsets for healthy Chinese Han adults in different regions with 3 flow cytometers (FACSCanto™II, FACSLyric™, FACSCalibur™) in each center using a single-platform detection method. The influencing factors of reference intervals, such as detection platform and method, age, and gender of the detection participant, were discussed. We found that there was no significant difference in the results from different instruments when using the standardized methods; the influence of gender on lymphocyte subsets was mainly in the percentage and absolute count of $CD3^-CD16^+CD56^+$ cells and the percentage of $CD4^+$ cells (%), while age mainly affected the ratio of $CD4^+/CD8^+$.

Methods and instruments

Compared with other studies, we found that the detection

Table 2 Reference interval of lymphocyte subsets for healthy Chinese Han adults

Lymphocyte subsets	Reference interval (P2.5–P97.5)		90% CI for P2.5		90% CI for P97.5	
	Lower	Higher	Lower	Higher	Lower	Higher
$CD3^-CD16^+CD56^+$ (cells/μL)						
Male and female	136.29	880.04	124.26	143.19	808.3	966.95
Male	153.58	966.95	122.79	181.43	847.74	1,113.63
Female	134.02	764.99	105.03	136.62	711.43	846.4
$CD3^-CD16^+CD56$ (%)	6.85	36.98	6.3	7.72	33.85	39.94
$CD3^-CD19^+$ (cells/μL)	91.53	498	82.94	98.58	454.18	517.37
$CD3^-CD19$ (%)	5.05	20.45	4.68	5.55	19.06	21.46
$CD3^+$ (cells/μL)	834.47	2,216.8	768.39	892.05	2,162.02	2,262.82
$CD3^+$ (%)	52.11	81.55	49.61	53.44	80.38	83.22
$CD3^+CD4^+$ (cells/μL)	395.36	1,264.17	366.95	417.07	1,184.22	1,316.34
$CD3^+CD4^+$ (%)						
Male and female	22.2	50.25	21.45	22.94	48.12	51.26
Male	21.92	45.96	20.31	22.61	44.64	49.92
Female	24.90	50.97	20.66	26.10	50.25	52.21
$CD3^+CD8^+$ (cells/μL)	269.47	1,059.43	244.89	290.85	985.15	1,125.65
$CD3^+CD8^+$ (%)	14.19	43.41	12.46	16.18	41.08	46.38
$CD4^+/CD8^+$ (years)						
18–59	0.6	2.88	0.55	0.63	2.7	3.15
18–39	0.55	2.09	0.49	0.61	1.96	2.37
40–59	0.65	3.28	0.57	0.73	2.88	3.73

CI, confidence interval.

method's influence and detection platform on the results cannot be ruled out. The cytometry-based absolute count methods for immune cells mainly include a single-platform method and a dual-platform method. To obtain known volume (such as 50 μL) absolute count of lymphocyte subsets in whole blood, single-platform refers to the use of known concentrations of microspheres to directly obtain the absolute count results of each lymphocyte subset, mainly including the use of BD Trucount tubes, and Beckman-Coulter's Flow-Count Fluorospheres. In contrast, the dual-platform method first obtains each lymphocyte subset percentage through flow cytometry and then multiplies it by the number of lymphocytes obtained by the hemocytometer (26,27). Compared with dual-platform detection used in previous research, the single-platform method is not affected by the blood cell counter's detection performance. It has fewer practical steps and more effortless operations. In recent years, the single-platform method has been applied more frequently (18,24). The detection platform mainly refers to the detection instruments used, including the flow cytometer and blood cell counter. In this study, to avoid the excessive influence of detection methods and platforms on the results, three flow cytometers common in clinical were used in the 3 centers. A standardized single-platform method was used. As a result, there was no significant difference in the detection results between the instruments, indicating that the results of lymphocyte subsets are comparable if the quality control and the detection method are consistent. In terms of detection methods, previous studies have shown that laboratories using the dual-platform method may have inter-chamber deviations due to the counting results obtained from different blood counters (27).

Still, this difference can be reduced by an optimized dual-platform method to achieve the same precision as the single platform method (28). At present, there is no comparative study on the use of different single-platform detection methods or the duplicated detection method with other instruments. However, compared to using various detection methods, the results from different flow cytometers using the same method may be more confidently interpreted. In addition, the flow cytometric single-platform method could be used to enumerate any cell and its subsets, including monocytes and granulocytes, but these require the use of relative antibody panels.

Gender

Many previous studies have shown that gender is one of the influencing factors of lymphocyte subsets. In this study, we found that women's $CD4^+$ cells (%) were higher than men's, while men's absolute counts of NK cells ($CD3^-CD16^+CD56^+$) were higher than women's, which is consistent with the results of some previous studies. Studies in South Korea (19), Qatar (21), Brazil (22), Italy (29), and Israel (30) have also observed gender differences in $CD4^+$ cells (%) and the absolute counts of NK cells. However, studies in Qatar (21), Brazil (22), Italy (29), and Israel (30) reported that there are gender differences in the absolute counts of $CD4^+$ cells, as well as in Singapore (15), Hong Kong (18), South Korea (19), and Italy (29). The results of gender differences in NK cells (%) reported in the Israeli (31) study were not observed in this study. In addition, this study did not observe the results of gender differences in $CD3^+$ cells (%), $CD3^+$ cell counts, and $CD4^+/CD8^+$ ratios reported in some studies (16,17,19,20,22,32). The inconsistency of these results may be attributed to differences in the inclusion criteria, sample size, and age distribution of the participants.

Age

The number of lymphocyte subsets presents different trends with age. This study showed that $CD3^+CD4^+$ cells increased slightly with age, while $CD3^+CD8^+$ cells showed a downward trend. The $CD4^+/CD8^+$ ratio of people over 40 was significantly higher than that of people under 40. As far as $CD4^+$ cells are concerned, reports in Malawi (5), Spain (33), Israel (31), and Cuba (29) showed the same age change trend as this research, while the study results of Hong Kong (16), on the contrary, demonstrated that $CD4^+$ cells decreased with age. Studies in Singapore (13), Germany (14), Israel (31), and Cuba (29) are consistent with the results of this study in terms of $CD8^+$ cells, while studies in Spain (33) showed that $CD8^+$ cells were positively correlated with age. In terms of the $CD4^+/CD8^+$ ratio, the studies of Germany (14), Spain (33), and Cuba (29) had the same age trends as this study. The possible explanation for the change of lymphocyte subsets with age in this study is that the telomere length of $CD3^+CD4^+$ cells is relatively constant, and less affected by age. Simultaneously, the telomere length of $CD3^+CD8^+$ cells is more susceptible to erosion with immune aging. Its sensitivity to telomerase induction also decreases with age; thereby, its number is reduced with age (30).

Races and regions

People of different races and regions may have differences in the number of lymphocyte subsets due to genetic factors, ecological environment, lifestyle, and nutritional status. In our study, except for the differences in $CD19^+$ cells in Guangzhou, the differences of other indicators of lymphocyte subsets in Shanghai, Beijing, and Guangzhou were not found to be statistically significant. Although there was no gender difference in $CD19^+$ cells, we still could not rule out that this difference in Guangzhou was caused by gender bias in the enrollment. Horizontal comparison of T lymphocyte subsets of different races in China showed that the absolute counts of $CD3^+$, $CD4^+$, and $CD8^+$ T cells in the Guangxi Han population were slightly higher than those in the Guangxi Zhuang population. Still, the difference is not statistically significant (34). The T lymphocyte subsets tests using the same Trucount tube showed differences in T lymphocyte subsets between the Han and other ethnic groups in China, such as the Yunnan Dai population, the Sichuan Yi population, and the Hunan Miao population, were not statistically significant (34).

Similarly, Brazil's analysis of lymphocyte subsets of people in different regions of the country showed that except for $CD3^+$ and $CD4^+$ cells with statistical differences in particular areas, the differences in lymphocyte subsets between most places were not statistically significant (35). A horizontal comparison of the results of this study with similar single-platform studies found that the absolute counts of lymphatic subpopulations studied in Qatar (19), especially T lymphocyte subsets, were higher than the results of this study. However, the results of Singapore (13) showed that the absolute counts and percentages of the lymphocyte subgroups, especially the T cell subgroups, and the $CD4^+/CD8^+$ ratio were similar to this study. In contrast,

the research results on T cell subgroups in India (36) and Brazil (35) showed that the absolute counts of $CD4^+$T cells were higher than in this study. The differences between the results of these studies may be related to differences in ethnic composition. For example, nearly 80% of the participants in the Singapore study (13) were of Chinese descent, which was similar to the ethnic composition of this study, while the ethnic composition of participants in other studies was entirely different from this study, the study participants in Qatar (19) were all Arabs, and the studies in Brazil (35) and India (36) were all multi-ethnic populations. These differences may also be due to the significant differences in the age and gender distribution of the participants in these studies. In particular, 90% of the participants in Qatar (19) study were under 45 years old, and 64% were male; more than 85% of the participants in the Indian (36) study were under 40 years old, and more than 60% of the participants in the Brazil (35) study were men. These confounding factors make it difficult for us to simply attribute the differences between lymphocyte subgroups studied worldwide to differences in race. The differences in lymphocyte subsets between different races need to be confirmed through an international multi-center study that strictly controls various confounding factors.

Limitations of our study include strict enrollment criteria, restriction of age, and rough lymphocytic grouping. The strict selection criteria lead to the high elimination rate at the screening stage, and the sample size analyzed was relatively small. In this study, children, juveniles, and people over 60 years old were not included, and we only distinguished Th/Tc cells, B cells, and NK cells. Moreover, despite having little effect on the reference intervals, gender bias may have existed in Guangzhou.

Conclusions

Despite the limitations, the present study established reference intervals of healthy Chinese Han adults' lymphocyte subsets, considering detection methods and instruments, gender, and age. This study was the first multi-center study, simultaneously using 3 flow cytometers in each center with a single-platform method to establish reference intervals of lymphocyte subsets. Gender and age were the main influencing factors. Although the ethnicity and region of the participants may also have an impact, more research is needed to clarify the extent of such influence. Therefore, it is recommended that laboratories and clinics in various regions cooperate to establish reference intervals of lymphocyte subsets used for cellular immune monitoring with a similar and standardized method to facilitate more accurate and reasonable clinical decision-making. Future research will expand the age range and establish reference intervals for more defined immune cell subsets than T cells, B cells, and NK cells.

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Footnote

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Data Sharing Statement: Available at https://dx.doi.org/10.21037/atm-21-4031

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The ethics committees of each center approved this study [ethics approval number: Zhongshan Hospital Affiliated to Fudan University, B2018-115; Peking University First Hospital, (2018) Research No. (145); Guangdong Provincial Hospital of Traditional Chinese Medicine, BF2018-087-01], and

all participants provided written informed consent. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013).

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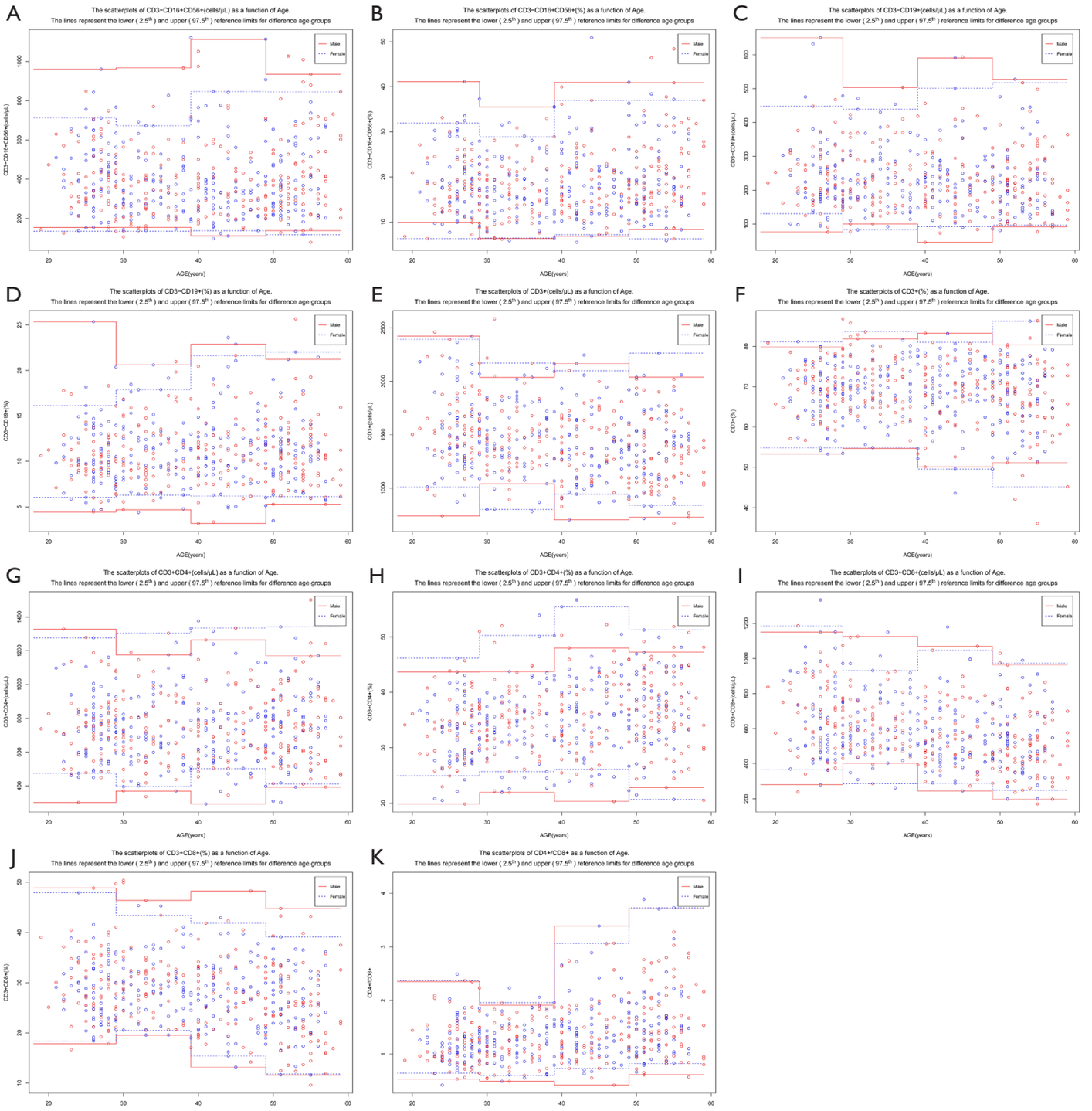

Figure S1 Age and gender distribution (Straight lines represent reference intervals for different age groups: Upper limit P97.5 and lower limit P2.5). (A) Absolute count of $CD3^-CD16^+CD56^+$ cells; (B) $CD3^-CD16^+CD56^+$ cells (%); (C) absolute count of $CD3^-CD19^+$ cells; (D) $CD3^-CD19^+$ cells (%); (E) absolute count of $CD3^+$ cells; (F) $CD3^+$ cells (%); (G) absolute count of $CD3^+CD4^+$ cells; (H) $CD3^+CD4^+$ cells (%); (I) absolute count of $CD3^+CD8^+$ cells; (J) $CD3^+CD8^+$ cells (%); (K) $CD4^+/CD8^+$ ratio.

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Table S1 Results comparison of quality control blood in 3 centers

Instrument	Type	Project	Center	QC Blood 1	QC Blood 2	QC Blood 3	QC Blood 4	QC Blood 5	Mean±2SD	Range	Number of out of control[a]
FACSCalibur®	High	CD3+ (%)	01	71	72	71	72	71	71.2±1.12	60.9-80.9	1
			02	71	71	71	71	71			
			03	72	71	70	71	72			
BD FACSCanto II	High	CD3+ (%)	01	71.2	71.01	71.26	71.72	71.91	71.763±1.3768	60.9-80.9	1
			02	71.99	70.37	71.11	71.87	72			
			03	72.93	71.97	71.92	72.89	72.3			
BD FACSLyric	High	CD3+ (%)	01	72.48	71.07	68.48	72.28	71.29	70.216±3.2361	60.9-80.9	0
			02	71.67	70.8	70.01	71.81	69.85			
			03	67.45	70.1	67.69	68.5	69.76			
FACSCalibur®	Low	CD3+ (%)	01	52	53	53	53	55	53.0±2.00	43.0-63.0	0
			02	53	52	53	53	54			
			03	54	52	51	54	53			
BD FACSCanto II	Low	CD3+ (%)	01	52.86	53.8	53.33	52.01	53.06	53.091±1.5255	43.0-63.0	0
			02	52.32	52.79	52.67	53.06	54.51			
			03	52.61	53.67	52.82	52.28	54.57			
BD FACSLyric	Low	CD3+ (%)	01	54.91	54.37	53.99	54.2	53.67	52.875±2.6101	43.0-63.0	0
			02	52.28	52.48	53.55	52.34	53.75			
			03	50.4	51.51	52.48	52.06	51.14			
FACSCalibur®	High	CD3+ (cells/ul)	01	1115	1271	1201	1191	1257	1237.2±115.74	1003.4-1505.1	1
			02	1240	1294	1274	1336	1256			
			03	1246	1145	1213	1292	1227			
BD FACSCanto II	High	CD3+ (cells/ul)	01	1158.22	1260.92	1271.86	1239.44	1349.97	1224.281±102.4005	1003.4-1505.1	1
			02	1196.53	1188.73	1157.8	1274.72	1237.35			
			03	1226.49	1190.55	1195.07	1185.63	1230.94			
BD FACSLyric	High	CD3+ (cells/ul)	01	1197	1252	1272	1161	1284	1205.3±114.44	1003.4-1505.1	0
			02	1147	1240	1195	1260	1262			
			03	1236	1122	1194	1117	1140			
FACSCalibur®	Low	CD3+ (cells/ul)	01	628	670	650	620	636	640.7±38.37	515.5-806.3	0
			02	665	636	655	634	648			
			03	636	609	609	651	664			
BD FACSCanto II	Low	CD3+ (cells/ul)	01	657.24	647.54	646.97	634.42	635.24	626.990±81.0412	515.5-806.3	1
			02	639.11	637.78	621.13	644.31	710.61			
			03	549.78	577.91	567.4	590.84	644.57			

Table S1 (*continued*)

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Table S1 (*continued*)

Instrument	Type	Project	Center	QC Blood 1	QC Blood 2	QC Blood 3	QC Blood 4	QC Blood 5	Mean±2SD	Range	Number of out of control[a]
BD FACSLyric	Low	CD3+ (cells/ul)	01	660	668	676	643	625	636.8±94.13	515.5-806.3	0
			02	685	666	662	645	709			
			03	550	550	601	621	591			
FACSCalibur*	High	CD3−CD19+ (%)	01	16	15	16	16	16	15.9±0.92	12.1-20.1	1
			02	16	16	16	16	16			
			03	16	16	17	15	16			
BD FACSCanto II	High	CD3−CD19+ (%)	01	15.75	15.87	15.81	16.36	16.44	15.781±0.9743	12.1-20.1	1
			02	15.96	15.86	15.15	16.05	14.76			
			03	14.97	15.65	16.13	15.73	16.22			
BD FACSLyric	High	CD3−CD19+ (%)	01	14.53	16.45	15.35	16.09	16.29	15.713±1.7501	12.1-20.1	1
			02	16.19	16.17	17.54	16.08	16.43			
			03	14.89	14.7	14.57	15.55	14.87			
FACSCalibur*	Low	CD3−CD19+ (%)	01	24	24	23	24	22	23.7±1.77	17.9-29.9	1
			02	24	24	24	23	23			
			03	26	24	24	23	24			
BD FACSCanto II	Low	CD3−CD19+ (%)	01	24.33	25.14	24.19	24.19	25.08	24.463±1.3822	17.9-29.9	0
			02	23.95	23.84	23.33	24.71	23.47			
			03	24.95	25.08	23.9	25.28	25.51			
BD FACSLyric	Low	CD3−CD19+ (%)	01	24.45	24.26	24.42	24.04	23.98	23.702±1.4892	17.9-29.9	0
			02	22.28	24.83	24.17	22.88	22.53			
			03	23.5	24.06	23.37	23.64	23.12			
FACSCalibur*	High	CD3−CD19+ (cells/ul)	01	258	263	263	269	326	281.3±36.23	197.1-372.5	1
			02	286	288	288	295	282			
			03	278	264	300	291	268			
BD FACSCanto II	High	CD3−CD19+ (cells/ul)	01	260.2	287.35	297.37	288.31	332.14	272.231±45.4752	197.1-372.5	1
			02	267.19	263.02	251.29	286.84	245.62			
			03	254.1	259.73	256.23	259.3	274.77			
BD FACSLyric	High	CD3−CD19+ (cells/ul)	01	248	294	293	254	318	271.1±48.61	197.1-372.5	0
			02	264	274	305	273	293			
			03	260	240	249	260	242			

Table S1 (*continued*)

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Table S1 (*continued*)

Instrument	Type	Project	Center	QC Blood 1	QC Blood 2	QC Blood 3	QC Blood 4	QC Blood 5	Mean±2SD	Range	Number of out of control[a]
FACSCalibur*	Low	CD3–CD19+ (cells/ul)	01	286	295	277	279	239	284.1±40.15	186.9-409.2	1
			02	304	305	286	278	263			
			03	260	302	310	273	304			
BD FACSCanto II	Low	CD3–CD19+ (cells/ul)	01	292.82	297.22	289.9	309.3	299.29	290.653±27.1495	186.9-409.2	0
			02	291.73	291.11	266.28	308.06	295.19			
			03	274.57	275.12	268.38	301.74	299.09			
BD FACSLyric	Low	CD3–CD19+ (cells/ul)	01	287	273	304	280	281	284.5±29.92	186.9-409.2	1
			02	295	317	289	288	291			
			03	267	271	270	294	261			
FACSCalibur*	High	CD3+CD4+ (%)	01	46	46	45	47	46	45.7±1.63	39.5-52.5	1
			02	45	45	47	46	46			
			03	46	45	45	44	46			
BD FACSCanto II	High	CD3+CD4+ (%)	01	45.23	44.58	47.15	48	47.93	46.275±2.1281	39.5-52.5	0
			02	45.73	44.56	46.26	46.72	46.13			
			03	47	46.26	45.7	47.16	45.72			
BD FACSLyric	High	CD3+CD4+ (%)	01	45.62	44.97	45.38	44.63	46.15	45.186±2.3069	39.5-52.5	1
			02	46.23	46.4	45.33	45.57	46.28			
			03	43.22	44.89	42.25	44.94	45.93			
FACSCalibur*	Low	CD3+CD4+ (%)	01	12	13	13	13	14	12.4±1.26	8.3-16.3	1
			02	12	12	12	12	12			
			03	12	12	12	12	13			
BD FACSCanto II	Low	CD3+CD4+ (%)	01	12.84	12.92	12.73	12.06	12.2	12.332±0.9839	8.3-16.3	0
			02	12.05	12.39	11.56	11.87	12.1			
			03	12.53	12.44	12.52	13.25	11.52			
BD FACSLyric	Low	CD3+CD4+ (%)	01	13.31	11.02	12.62	12.26	11.09	11.793±1.3233	8.3-16.3	1
			02	12.31	11.02	11.75	11.5	11.68			
			03	12.16	11.86	11.04	11.4	11.88			
FACSCalibur*	High	CD3+CD4+ (cells/ul)	01	712	844	808	756	718	784.7±100.09	651.0-976.5	0
			02	795	805	862	864	804			
			03	766	703	784	769	780			

Table S1 (*continued*)

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Table S1 (*continued*)

Instrument	Type	Project	Center	QC Blood 1	QC Blood 2	QC Blood 3	QC Blood 4	QC Blood 5	Mean±2SD	Range	Number of out of control[a]
BD FACSCanto II	High	CD3+CD4+ (cells/ul)	01	724.44	775.38	796.97	813.21	832.15	781.367±63.0512	651.0-976.5	0
			02	754.39	766.68	739.23	822	818.02			
			03	782.74	762.34	793.51	757.37	782.08			
BD FACSLyric	High	CD3+CD4+ (cells/ul)	01	728	781	823	729	763	773.2±97.52	651.0-976.5	0
			02	728	839	760	826	846			
			03	832	704	770	715	754			
FACSCalibur*	Low	CD3+CD4+ (cells/ul)	01	152	169	154	147	162	149.9±21.45	92.0-214.7	0
			02	153	143	156	140	158			
			03	150	131	130	147	157			
BD FACSCanto II	Low	CD3+CD4+ (cells/ul)	01	164.64	158.19	156.37	139.94	146.46	144.512±24.4540	92.0-214.7	0
			02	147.61	148.04	140.81	140.3	163.24			
			03	123.78	131.48	128.15	141.6	137.07			
BD FACSLyric	Low	CD3+CD4+ (cells/ul)	01	163	147	159	148	129	142.3±27.94	92.0-214.7	0
			02	160	139	150	139	157			
			03	127	120	125	130	141			
FACSCalibur*	High	CD3+CD8+ (%)	01	22	24	22	24	23	22.3±1.63	16.4-30.4	2
			02	22	23	21	22	22			
			03	22	22	22	22	22			
BD FACSCanto II	High	CD3+CD8+ (%)	01	26.39	25.66	25.38	24.88	25.27	24.311±4.0389	16.4-30.4	0
			02	22.46	22.12	21.15	20.7	22.19			
			03	24.58	25.15	25.56	26.84	26.33			
BD FACSLyric	High	CD3+CD8+ (%)	01	21.1	22.59	21	23.64	22.66	21.221±2.5045	16.4-30.4	0
			02	22.51	21.97	21.32	21.27	20.75			
			03	19.97	20.62	19.29	19.83	19.79			
FACSCalibur*	Low	CD3+CD8+ (%)	01	33	33	36	32	37	34.5±2.91	30.1-44.1	0
			02	34	34	34	34	34			
			03	36	34	34	37	35			
BD FACSCanto II	Low	CD3+CD8+ (%)	01	40.59	41.7	39.42	39.65	40.94	38.451±5.8891	30.1-44.1	0
			02	33.62	34.39	35.02	35.2	36.18			
			03	38.53	40.31	38.69	38.92	43.61			

Table S1 (*continued*)

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Table S1 (*continued*)

Instrument	Type	Project	Center	QC Blood 1	QC Blood 2	QC Blood 3	QC Blood 4	QC Blood 5	Mean±2SD	Range	Number of out of control[a]
BD FACSLyric	Low	CD3+CD8+ (%)	01	36.45	37.84	36.32	35.49	37.22	34.909±3.4045	30.1-44.1	0
			02	34.56	33.63	35.08	32.01	35.32			
			03	32.73	34.82	35.58	34.04	32.54			
FACSCalibur®	High	CD3+CD8+ (cells/ul)	01	344	432	404	396	365	385.1±51.97	298.5-529.4	0
			02	398	420	385	417	375			
			03	372	345	380	376	368			
BD FACSCanto II	High	CD3+CD8+ (cells/ul)	01	422.59	446.27	428.97	421.41	438.8	410.266±67.5728	298.5-529.4	1
			02	370.6	380.59	338.06	364.25	393.52			
			03	409.34	414.45	443.79	430.99	450.36			
BD FACSLyric	High	CD3+CD8+ (cells/ul)	01	337	392	381	386	375	362.9±54.72	298.5-529.4	0
			02	354	397	357	386	379			
			03	384	323	352	315	325			
FACSCalibur®	Low	CD3+CD8+ (cells/ul)	01	405	424	437	369	437	416.9±56.43	339.1-586.2	0
			02	429	398	426	399	435			
			03	463	369	383	443	436			
BD FACSCanto II	Low	CD3+CD8+ (cells/ul)	01	520.37	510.43	484.32	460.11	491.7	450.540±95.5245	339.1-586.2	0
			02	411.92	411.08	426.62	416.15	488.24			
			03	380.58	425.92	396.11	415.8	518.75			
BD FACSLyric	Low	CD3+CD8+ (cells/ul)	01	447	506	457	429	431	421.4±90.72	339.1-586.2	0
			02	448	424	447	386	476			
			03	342	351	403	389	385			
FACSCalibur®	High	CD3–CD16+CD56 (%)	01	11	12	11	11	12	11.5±1.49	4.7-18.7	2
			02	12	12	12	12	12			
			03	10	12	11	12	10			
BD FACSCanto II	High	CD3–CD16+CD56 (%)	01	12.13	11.83	12.44	11.49	11.4	11.387±1.4058	4.7-18.7	0
			02	10.97	12.02	12.14	10.38	12.03			
			03	10.18	10.93	10.67	11.35	10.84			

Table S1 (*continued*)

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Table S1 (*continued*)

Instrument	Type	Project	Center	QC Blood 1	QC Blood 2	QC Blood 3	QC Blood 4	QC Blood 5	Mean±2SD	Range	Number of out of control[a]
BD FACSLyric	High	CD3–CD16+CD56 (%)	01	10.23	11.23	12.75	10.63	11.53	11.210±1.3494	4.7-18.7	1
			02	11.23	11.42	11.02	9.87	11.89			
			03	11.32	10.84	11.33	11.39	11.47			
FACSCalibur®	Low	CD3–CD16+CD56 (%)	01	22	21	21	20	21	21.0±1.69	16.5-26.5	1
			02	20	21	21	21	20			
			03	20	21	23	22	21			
BD FACSCanto II	Low	CD3–CD16+CD56 (%)	01	22.68	20.67	20.21	22.6	21.47	21.375±1.5324	16.5-26.5	0
			02	21.63	22.02	21.65	21.69	20.76			
			03	20.9	20.61	21.81	21.59	20.34			
BD FACSLyric	Low	CD3–CD16+CD56 (%)	01	20.05	19.32	19.97	19.77	20.63	20.537±1.8588	16.5-26.5	1
			02	22.53	20.41	19.88	21.23	20.51			
			03	22.25	20.76	20.83	19.32	20.6			
FACSCalibur®	High	CD3–CD16+CD56 (cells/ul)	01	167	199	176	188	245	199.9±41.52	109.7-304.3	1
			02	200	214	211	219	207			
			03	186	199	187	224	177			
BD FACSCanto II	High	CD3–CD16+CD56 (cells/ul)	01	200.43	214.26	233.98	202.52	230.41	196.408±37.8831	109.7-304.3	0
			02	183.61	199.37	201.32	185.6	200.19			
			03	172.79	181.42	169.53	187	183.69			
BD FACSLyric	High	CD3–CD16+CD56 (cells/ul)	01	174	201	243	168	225	193.6±41.31	109.7-304.3	1
			02	183	193	192	168	212			
			03	197	177	194	190	187			
FACSCalibur®	Low	CD3–CD16+CD56 (cells/ul)	01	253	258	259	230	235	251.9±37.17	198.7-337.5	1
			02	252	269	250	251	228			
			03	214	265	286	268	261			

Table S1 (*continued*)

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Table S1 (*continued*)

Instrument	Type	Project	Center	QC Blood 1	QC Blood 2	QC Blood 3	QC Blood 4	QC Blood 5	Mean±2SD	Range	Number of out of control[a]
BD FACSCanto II	Low	CD3–CD16+CD56 (cells/ul)	01	273.02	244.39	242.17	288.94	256.24	254.198±34.5960	198.7-337.5	1
			02	263.41	268.93	247.09	270.46	261.13			
			03	230.05	226.06	244.98	257.6	238.5			
BD FACSLyric	Low	CD3–CD16+CD56 (cells/ul)	01	235	217	248	230	242	246.8±39.92	198.7-337.5	1
			02	299	261	238	267	265			
			03	253	234	241	240	232			

*, Center 01 used BD Calibur. [a], the number of test results outside the range of mean ±2 SD. SD, standard deviation.

Table S2 Nested ANOVA for the influence of gender, age, and region on the value of lymphocyte subsets

		Df	Sum. Sq	Mean. Sq	F-value	P-value (>F)
CD-CD16+CD56+ (cells/ul)	Gender	1	721376.559	721376.559	20.454	0.000*
	Gender: Age	6	290145.333	48357.556	1.371	0.224
	Gender: Age: Region	16	549740.340	34358.771	0.974	0.484
	Residual	472	16646594.856	35268.209		
CD3–CD16+CD56+ (%)	Gender	1	1367.064	1367.064	24.847	0.000*
	Gender: Age	6	634.815	105.803	1.923	0.075
	Gender: Age: Region	16	877.862	54.866	0.997	0.458
	Residual	472	25969.309	55.020		
CD3–CD19+ (cells/ul)	Gender	1	66117.489	66117.489	7.068	0.008*
	Gender: Age	6	51359.039	8559.840	0.915	0.484
	Gender: Age: Region	16	338722.392	21170.150	2.263	0.004*
	Residual	472	4415329.103	9354.511		
CD3–CD19+ (%)	Gender	1	217.203	217.203	16.642	0.000*
	Gender: Age	6	105.397	17.566	1.346	0.235
	Gender: Age: Region	16	332.624	20.789	1.593	0.067
	Residual	472	6160.338	13.052		
CD3+ (cells/ul)	Gender	1	34097.462	34097.462	0.297	0.586
	Gender: Age	6	3340684.402	556780.734	4.858	0.000*
	Gender: Age: Region	16	2049239.693	128077.481	1.117	0.335
	Residual	472	54098967.663	114616.457		

Table S2 (*continued*)

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Table S2 (*continued*)

		Df	Sum. Sq	Mean. Sq	F-value	P-value (>F)
CD3+ (%)	Gender	1	478.127	478.127	8.212	0.004*
	Gender: Age	6	582.306	97.051	1.667	0.127
	Gender: Age: Region	16	851.807	53.238	0.914	0.553
	Residual	472	27482.167	58.225		
CD3+CD4+ (cells/ul)	Gender	1	387191.943	387191.943	8.371	0.004*
	Gender: Age	6	142770.685	23795.114	0.514	0.798
	Gender: Age: Region	16	888584.074	55536.505	1.201	0.263
	Residual	472	21832178.677	46254.616		
CD3+CD4+ (%)	Gender	1	1394.010	1394.010	33.066	0.000*
	Gender: Age	6	957.132	159.522	3.784	0.001*
	Gender: Age: Region	16	924.095	57.756	1.370	0.152
	Residual	472	19898.991	42.159		
CD3+CD8+ (cells/ul)	Gender	1	143317.143	143317.143	4.020	0.046*
	Gender: Age	6	1722187.283	287031.214	8.052	0.000*
	Gender: Age: Region	16	574811.822	35925.739	1.008	0.447
	Residual	472	16826332.856	35649.010		
CD3+CD8 (%)	Gender	1	167.851	167.851	3.473	0.063
	Gender: Age	6	1447.980	241.330	4.993	0.000*
	Gender: Age: Region	16	770.949	48.184	0.997	0.459
	Residual	472	22811.758	48.330		
CD4+/CD8+	Gender	1	3.283	3.283	11.220	0.001*
	Gender: Age	6	14.394	2.399	8.200	0.000*
	Gender: Age: Region	16	5.956	0.372	1.272	0.210
	Residual	472	138.088	0.293		

* The difference is statistically significant. ANOVA, analysis of variance; Df, degrees of freedom.

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Table S3 Z-test and difference analysis grouped by Gender·1

	Male		Female		Z	Z standard (Z*)	Z difference (Z-Z*)
	Mean	SD	Mean	SD			
N	233	233	263	263			
CD3–CD16+CD56+ (cells/ul)	440.181	203.701	363.768	173.203	4.47	4.31	0.16†
CD3–CD16+CD56+ (%)	20.591	7.935	17.264	7.010	4.92	4.31	0.61†
CD3–CD19+ (cells/ul)	222.636	105.487	245.769	92.129	-2.59	4.31	-1.72
CD3–CD19+ (%)	10.375	3.902	11.701	3.420	-4	4.31	-0.31
CD3+ (cells/ul)	1439.807	345.823	1456.420	348.076	-0.53	4.31	-3.78
CD3+ (%)	67.723	7.711	69.690	7.598	-2.86	4.31	-1.45
CD3+CD4+ (cells/ul)	715.491	213.929	771.473	216.195	-2.89	4.31	-1.42
CD3+CD4+ (%)	33.518	6.574	36.877	6.698	-5.63	4.31	1.32†
CD3+CD8+ (cells/ul)	608.724	199.705	574.665	194.099	1.92	4.31	-2.39
CD3+CD8+ (%)	28.616	7.429	27.450	6.831	1.81	4.31	-2.5
CD4+/CD8+	1.297	0.572	1.460	0.562	-3.2	4.31	-1.11

* Statistic Z with a “critical” value purposed by “Harris and Boyd”. † The difference is statistically significant. SD, standard deviation.

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