

Characteristics of and reference ranges for peripheral blood lymphocytes and CD4+ T cell subsets in healthy adults in Shanxi Province, North China

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Abstract

Objective: To guide clinical decision making, race-, age- and gender-specific reference ranges for lymphocytes and CD4+ T-cell subsets are required.

Methods: Single platform flow cytometry to determine reference intervals for lymphocyte subpopulations and CD4+ T-cell subsets in 196 healthy Han Chinese adults.

Results: The frequencies and absolute numbers of B cells were slightly lower in Han Chinese individuals of the Shanxi region than in individuals from Hong Kong, Germany and Singapore, while percentages and absolute numbers of NK cells were slightly higher compared with individuals from Hong Kong. CD4+/CD8+ T-cell ratios, CD4+ T cell percentages and Th2 cell counts were higher, while frequencies and numbers of CD8+ T cells, numbers of NK cells and percentages of Th1 cells were lower, in females compared with males. CD4+ T cell percentages, CD4+/CD8+ T-cell ratios, numbers of CD8+ T cells and Treg cells, and Th17/Treg cell ratios differed by age.

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Conclusion: We established lymphocyte and CD4+ T-cell subset reference intervals for healthy Han Chinese adults of the Shanxi region. Ethnicity, gender and age affected lymphocyte subset composition.

Keywords

Lymphocyte subsets, CD4+ T cell subsets, flow cytometry, reference ranges, ethnicity, age, gender

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Introduction

The innate and adaptive immune systems serve to protect the body from damage.¹ Peripheral blood lymphocytes play key roles in maintaining immune homeostasis, and contribute to adaptive immune responses through both humoral and cellular immunity.² CD4+ T cells are activated following stimulation, and can be subdivided into helper T cells (Th cells) including the effector Th1 and Th2 subpopulations, the more recently discovered Th17 cells, and regulatory T cells (Treg cells).¹⁻³ Different effector and regulatory subsets carry out a variety of functions during immune responses, ranging from activation of cytotoxic T cells and B cells to induction of Treg cells; the latter play important roles in suppression of immune responses.⁴ Activation or dysregulation of lymphocyte subsets can contribute to the onset or progression of diseases including leukemia, allergy, immunodeficiency syndromes and autoimmune diseases.^{5,6} Therefore, analysis of lymphocyte subpopulations can offer an effective means to understand disease pathogenesis and progression, to assess the immune status of patients, and to evaluate treatment outcomes.

Flow cytometry is typically used to analyze lymphocyte subsets in the laboratory.⁷ However, to evaluate the immune status of

patients, it is necessary to establish reference ranges in healthy individuals, carefully matched for gender, age, and ethnicity.⁸ Several studies have been carried out to identify normal reference ranges for lymphocyte subsets in healthy Chinese adults.⁹⁻¹¹ However, reference ranges for lymphocyte subsets and CD4+ T cell subsets in healthy Han Chinese individuals of the Shanxi region have not been reported previously. Thus, this study aimed to establish reference intervals for the absolute numbers and percentages of peripheral blood lymphocytes and CD4+ T cell subsets in healthy Han Chinese individuals of the Shanxi region, and to assess differences in these ranges associated with age, race and sex.

Methods

Study population

Healthy Han Chinese individuals who visited the Second Hospital of Shanxi Medical University for regular medical checkups were enrolled in the study. All individuals lived in the Shanxi area. Exclusion criteria included use of steroids or immunosuppressants and history of serious medical problems including infections (e.g., human immunodeficiency virus or hepatitis B virus) or chronic noninfectious conditions

(e.g., autoimmune diseases, allergies, cancer, chronic renal disease and diabetes mellitus). To examine changes in cell subtypes associated with ages, individuals were divided into five age strata (20–30 years; 31–40 years; 41–50 years; 51–60 years and 61–70 years). We also compared levels of lymphocyte subpopulations and CD4⁺ T cell subsets between males and females. All participants provided written informed consent and the study protocol was approved by the ethics committee of the Second Hospital of Shanxi Medical University (2016KY007). After fasting for 10 to 12 hours, blood samples were collected from the antecubital vein into tubes containing ethylenediaminetetraacetic acid (EDTA) and heparin as anticoagulants.

Analysis of lymphocyte subsets

To determine percentages and numbers of T cells (CD3⁺CD19⁻), B cells (CD3⁻CD19⁺), CD4⁺ T cells (CD3⁺CD4⁺), CD8⁺ T cells (CD3⁺CD8⁺), and natural killer (NK) cells (CD3⁻CD16⁺CD56⁺), peripheral blood samples (2 mL) from each subject were collected. For immunofluorescence staining, 50 μ L of each blood sample were placed in TruCount tubes A and B. Then, 20 μ L of CD3-fluorescein isothiocyanate (FITC)/CD8-phycoerythrin (PE)/CD45-peridinin-chlorophyll protein (PerCP)/CD4-allophycocyanin (APC) antibodies (clones SK7/SK1/2D1/SK3, respectively) were added to tube A and 20 μ L of CD3-FITC/CD16 + 56-PE/CD45-PerCP/CD19-APC antibodies (clones SK7/B73.1/NCAM16.2/2D1/SJ25C1, respectively) were added to tube B. All antibodies were purchased from BD Biosciences (San Jose, CA, USA). After incubation at room temperature for 20 minutes in the dark, stained cells were washed with 1 \times FACS buffer and then incubated for 15 minutes in the dark. Data on 15,000 cells were acquired on a

FACSCanto instrument (BD Bioscience) and analyzed using MultiSET software.

Analysis of CD4⁺T cell subsets

To analyze Th1, Th2, and Th17 cells, 80 μ L of heparinized blood were stimulated with 10 μ L of phorbol myristate acetate, 10 μ L of ionomycin and 1 μ L of GolgiStop. The cells were incubated for 5 hours at 37°C and then divided into Tube A and Tube B. Both tubes were stained with human anti-CD4-FITC (clone SK3) in the dark for 30 minutes at room temperature, then cells were fixed and permeabilized in the dark at 4°C for 30 minutes. Subsequently, cells in Tube A were stained with anti-IL-17-PE (clone SCPL1362) and anti-interferon (IFN)- γ -APC (clone 4S.B3), and those in Tube B were stained with human anti-IL-4-PE (clone MP4-25D2) in the dark at room temperature for 30 minutes. Cells were washed with phosphate-buffered saline and examined by four-color flow cytometry. To analyze Treg cells, 80 μ L of anti-coagulated blood were stained with anti-CD4-FITC and anti-CD25-APC (clone 2A3) at room temperature in the dark for 30 minutes. Cells were fixed and permeabilized in the dark at 4°C for 30 minutes, then stained with human anti-FOXP3-PE (clone PCH101) in the dark at room temperature for 30 minutes. Cells were washed and examined by flow cytometry (FACSCalibur, BD Biosciences). Data were acquired on 10,000 cells, then gated and analyzed using Cell Quest software to determine the frequencies of CD4⁺ T cell subsets. Absolute numbers of CD4⁺ T cell subsets were calculated by multiplying the percentage of each subset by the absolute number of total CD4⁺ T cells. Cell types were defined as Th1 (CD4⁺IFN- γ ⁺), Th2 (CD4⁺ IL-4⁺), Th17 (CD4⁺IL-17⁺), or Treg (CD4⁺CD25⁺FOXP3⁺).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA) and SPSS Statistics for Windows, version 22.0 (SPSS Inc., Chicago, IL, USA). Continuous variables were reported as the mean \pm standard deviations (SD). The Kolmogorov–Smirnov test was used to assess normality. Reference limits were defined as the 95th percentile of observed data. Since the absolute numbers and percentage of CD4+ T cells were normally distributed, we used the Student's t-test to assess differences between lymphocyte subsets in men and women. For non-normally distributed lymphocyte subsets, comparisons among different age groups were

made using non-parametric tests. The Kruskal–Wallis H test was used to evaluate differences between age groups, Spearman's rank correlation was used to evaluate relationships between age and cell subsets, and the Mann–Whitney U test was used to evaluate the influence of gender on cell subsets. All tests were two-sided, and values of $P < 0.05$ were considered statistically significant.

Results

A total of 196 healthy Han Chinese individuals (mean \pm SD age: 43.22 \pm 13.46 years) were recruited in this study. Absolute numbers and percentages of lymphocyte subpopulations and CD4+ T cell subsets were

Table 1. Reference ranges for absolute counts and percentages of lymphocytes and CD4+ T cell subsets in healthy Han Chinese of the Shanxi district, China.

Parameter	Mean \pm SD	Median	Confidence interval*	Reference range**
Number of T cells/ μ L	1335 \pm 341	1312	1287–1383	746–2070
% T cells	70.39 \pm 8.52	71.22	69.19–71.59	56.57–83
Number of B cells/ μ L	205 \pm 87	188	193–218	78–429
% B cells	11.32 \pm 6.47	10.65	10.41–12.24	5.00–20.28
Number of CD4+T cells/ μ L	693 \pm 206	652	664–722	359–1122
% CD4+ T cells	37.17 \pm 7.56	37	36.11–38.24	22.93–52.05
Number of CD8+T cells/ μ L	510 \pm 201	479	482–539	193–976
% CD8+ T cells	27.15 \pm 7.87	26.40	26.05–28.26	12.93–46.19
CD4+/CD8+ T cell ratio	1.56 \pm 0.81	1.39	1.44–1.67	0.64–3.71
Number of NK cells/ μ L	312 \pm 164	285	289–336	101–708
% NK cells	16.53 \pm 6.83	15.61	15.57–17.50	5.80–31.05
Number of T+B+NK cells/ μ L	1883 \pm 461	1849	1818–1948	1049–2849
% T+B+NK cells	98.07 \pm 1.19	98.3	97.91–98.24	95.39–99.70
Number of Th1 cells/ μ L	117 \pm 67	106	108–127	14–275
% Th1 cells	26.41 \pm 45.93	16.93	19.95–32.89	4.85–162.21
Number of Th2 cells/ μ L	9 \pm 6	8	8–10	1–26
% Th2 cells	1.74 \pm 2.01	1.23	1.46–2.03	0.36–9.03
Number of Th17 cells/ μ L	7 \pm 4	7	7–9	1–18
% Th17 cells	1.58 \pm 2.54	1.09	1.22–1.94	0.32–8.10
Number of Treg cells/ μ L	31 \pm 15	30	29–33	5–64
% Treg cells	6.61 \pm 7.74	4.85	5.52–7.70	2.06–39.65
Th1/Th2 cell ratio	18.54 \pm 19.46	14.26	15.79–21.28	2.53–60.5
Th17/Treg cell ratio	0.26 \pm 0.17	0.21	0.24–0.28	0.06–0.70

*95% confidence interval of the sample mean. ** 2.5–97.5th percentile of reference range.

Th cells: Helper T cells, NK cells: natural killer cells.

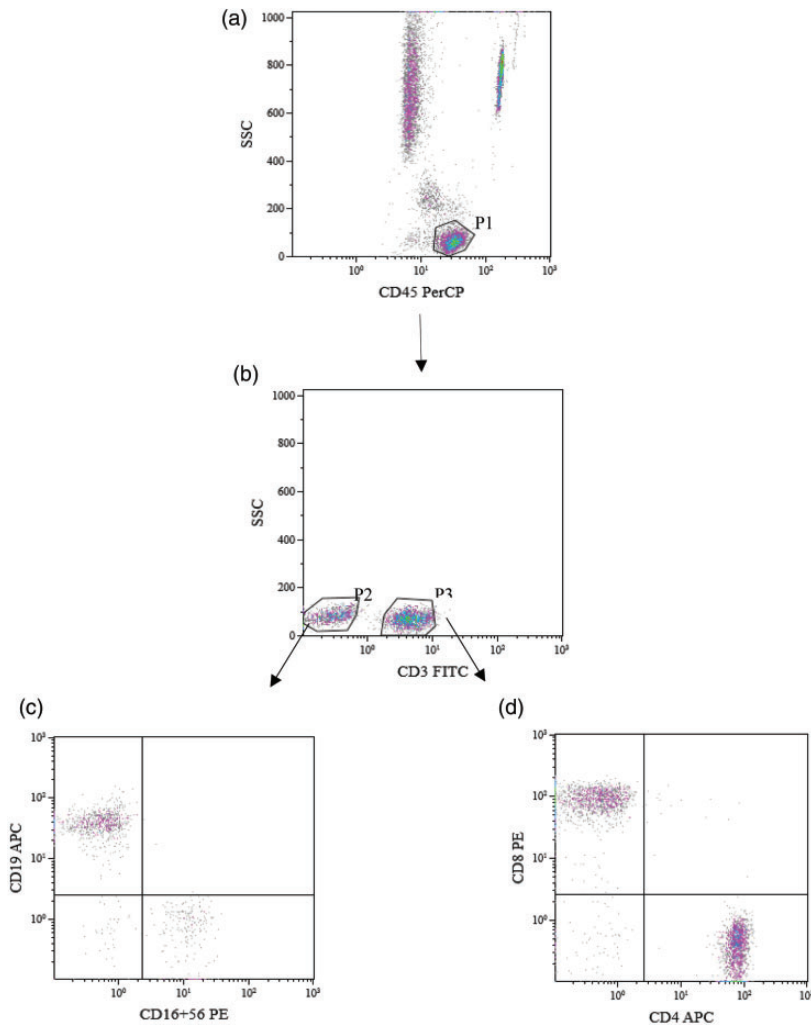


Figure 1a. Analysis of peripheral lymphocytes and CD4⁺ T cell subsets by flow cytometry. Gating strategy for T, B, and NK cells. (a): The gate was drawn to include lymphocytes (CD45⁺). (b) P2: CD3⁻ cells (CD3⁻) and P3: T cells (CD3⁺) (c): B cells (CD3⁻CD19⁺CD16-56⁻) and NK cells (CD3⁻CD19⁻CD16⁺CD56⁺). (d): Th cells (CD3⁺CD4⁺CD8⁻) and Ts cells (suppressor T lymphocytes) (CD3⁺CD4⁺CD8⁺).

reported as 95% confidence intervals, medians, mean \pm SD (Table 1). A flow cytometry dot plot of peripheral lymphocytes and CD4⁺ T cell subsets from a representative healthy individual is shown in Figure 1.

Gender-related differences in lymphocytes and CD4⁺ T cell subsets

To explore the influence of gender on cell subset frequency, data on the 77 males (mean \pm SD age: 42.84 \pm 12.94 years) and

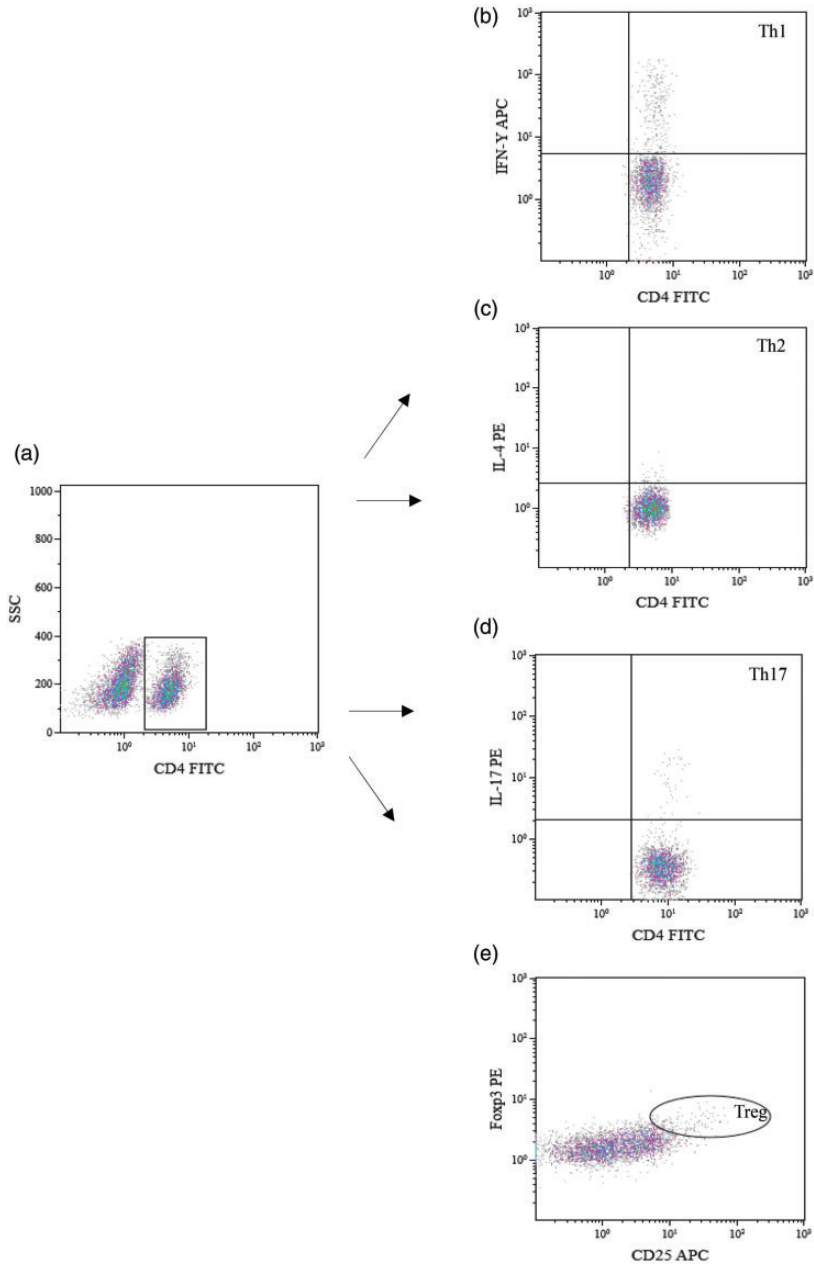


Figure 1b. CD4+T cell subsets. Gating strategy for Th1, Th2, Th17 and Treg cells. (a):T cells (CD4+). (b): Th1 (CD4+IFN- γ +). (c): Th2 (CD4+IL-4+). (d): Th17 (CD4+IL-17+). (e): Treg (CD4+CD25+FOXP3+).

Table 2. Reference ranges for absolute counts and percentages of lymphocytes and CD4+ T cell subsets in healthy Han Chinese men and women of the Shanxi district, China.

Parameter	Mean \pm SD		Reference range*		P-value
	Male (n = 77)	Female (n = 119)	Male (n = 77)	Female (n = 119)	
Age	42.82 \pm 12.94	43.49 \pm 13.82			
Number of T cells/ μ L	1391 \pm 339	1299 \pm 339	756–2106	672–2053	0.063
% T cells	70.14 \pm 7.63	70.56 \pm 9.07	51.93–85.31	58.00–83.00	0.742
Number of B cells/ μ L	206 \pm 95	205 \pm 82	62–474	92–395	0.905
% B cells	10.42 \pm 4.03	11.91 \pm 7.61	3.18–20.27	5.38–21.00	0.116
Number of CD4+ T cells/ μ L	694 \pm 202	693 \pm 209	345–1152	360–1122	0.993
% CD4+ T cells	35.37 \pm 7.90	38.34 \pm 7.12	22.85–52.86	25.00–52.00	0.007
Number of CD8+ T cells/ μ L	574 \pm 219	469 \pm 177	283–1083	160–865	0.001
% CD8+ T cells	28.94 \pm 8.06	26.00 \pm 7.56	16.81–50.10	10.00–40.98	0.01
CD4+/CD8+ T cell ratio	1.35 \pm 0.56	1.69 \pm 0.91	0.48–2.95	0.74–5.17	0.003
Number of NK cells/ μ L	364 \pm 198	279 \pm 128	100–908	88–553	0.001
% NK cells	17.66 \pm 6.99	15.81 \pm 6.66	5.11–34.77	6.00–31.00	0.064
T+B+NK cells/ μ L	1996 \pm 492	1810 \pm 426	1111–3239	1027–2724	0.005
% T+B+NK cells	98.22 \pm 1.04	97.98 \pm 1.27	95.58–99.80	95.20–99.70	0.152
Number of Th1 cells/ μ L	122 \pm 71	115 \pm 65	10–261	17–276	0.506
% Th1 cells	36.50 \pm 68.74	19.89 \pm 18.24	4.81–288.17	4.43–90.75	0.041
Number of Th2 cells/ μ L	8 \pm 5	10 \pm 6	0–24	1–28	0.042
% Th2 cells	1.89 \pm 2.38	1.65 \pm 1.74	0.29–11.67	0.41–4.37	0.429
Number of Th17 cells/ μ L	8 \pm 5	7 \pm 4	1–20	1–15	0.421
% Th17 cells	2.03 \pm 3.52	1.29 \pm 1.58	0.31–16.89	0.31–4.43	0.088
Number of Treg cells/ μ L	31 \pm 15	31 \pm 14	4–64	5–70	0.911
% Treg cells	7.96 \pm 10.40	5.74 \pm 5.22	2.25–50.61	2.02–26.03	0.086
Th1/Th2 cell ratio	20.87 \pm 17.15	17.03 \pm 20.74	3.90–78.89	1.85–55.20	0.178
Th17/Treg cell ratio	0.27 \pm 0.19	0.25 \pm 0.15	0.06–0.81	0.05–0.64	0.490

*2.5–97.5th percentile of reference range. Th cells: Helper T cells, NK cells: natural killer cells.

119 females (mean \pm SD age: 43.49 \pm 13.82 years) recruited in this study were compared (Table 2). Several parameters differed between men and women. Percentages of CD4+ T cells were higher in females than in males ($P=0.007$), while percentages and absolute numbers of CD8+ T cells as well as absolute numbers of NK cells and T+B+NK cells were lower in females than in males ($P=0.01$, $P=0.001$, $P=0.001$, and $P=0.005$, respectively). Furthermore, CD4+/CD8+ T cell ratios were lower in men compared with women ($P=0.003$). For other lymphocyte subsets, no statistically significant differences were observed between the two groups. Among CD4+ T

cell subsets, the percentage of Th1 cells was higher in men than in women ($P=0.041$), but numbers of Th2 cells were lower in men compared with women ($P=0.042$). No other significant differences in CD4+ T cell subsets were associated with gender.

Age-related differences in lymphocytes and CD4+ T cell subsets

To identify age-related differences among cell subsets, we stratified participants into five age groups. Table 3 shows the percentages and absolute numbers of lymphocyte subpopulations for each age group. Significant differences in the percentages

Table 3. Distribution of absolute counts and percentages of lymphocytes and CD4⁺ T cell subsets by age.

Parameter	Mean \pm SD					P-value
	20-30 (N = 45)	31-40 (N = 44)	41-50 (N = 41)	51-60 (N = 40)	61-70 (N = 26)	
Age stratum						
Number of T cells/ μ L	1443 \pm 290	1338 \pm 341	1295 \pm 382	1268 \pm 317	1310 \pm 373	0.100
% T cells	70.30 \pm 7.14	70.34 \pm 6.40	71.40 \pm 7.41	69.79 \pm 12.36	69.98 \pm 8.77	0.947
Number of B cells/ μ L	221 \pm 103	212 \pm 85	204 \pm 86	190 \pm 66	192 \pm 91	0.710
% B cells	10.75 \pm 3.65	11.08 \pm 3.60	11.63 \pm 4.38	12.70 \pm 12.11	10.14 \pm 4.05	0.519
Number of CD4 ⁺ T cells/ μ L	700 \pm 170	660 \pm 198	696 \pm 216	705 \pm 210	717 \pm 257	0.806
% CD4 ⁺ T cells	35.07 \pm 6.87	35.16 \pm 6.69	38.70 \pm 7.29	39.66 \pm 8.40	38.00 \pm 7.79	0.006
Number of CD8 ⁺ T cells/ μ L	563 \pm 158	542 \pm 197	465 \pm 225	463 \pm 209	510 \pm 200	0.006
% CD8 ⁺ T cells	27.90 \pm 5.63	28.74 \pm 7.29	25.54 \pm 8.66	26.14 \pm 9.15	27.29 \pm 8.63	0.090
CD4 ⁺ /CD8 ⁺ T cell ratio	1.32 \pm 0.40	1.35 \pm 0.60	1.73 \pm 0.76	1.81 \pm 1.04	1.67 \pm 1.12	0.004
Number of NK cells/ μ L	355 \pm 186	313 \pm 132	269 \pm 152	292 \pm 166	339 \pm 178	0.070
% NK cells	16.88 \pm 6.42	16.67 \pm 6.36	14.97 \pm 7.02	16.88 \pm 7.35	17.64 \pm 7.28	0.492
T+B+NK cells/ μ L	2040 \pm 459	1894 \pm 409	1807 \pm 467	1773 \pm 458	1884 \pm 503	0.092
% T+B+NK cells	97.88 \pm 1.25	98.08 \pm 0.95	98.05 \pm 1.18	98.51 \pm 1.07	97.78 \pm 1.47	0.076
Number of Th1 cells/ μ L	106 \pm 61	114 \pm 71	114 \pm 64	131 \pm 65	131 \pm 78	0.381
% Th1 cells	24.19 \pm 40.72	29.15 \pm 34.74	23.11 \pm 28.30	19.07 \pm 7.79	42.17 \pm 98.47	0.479
Number of Th2 cells/ μ L	10 \pm 6	8 \pm 6	8 \pm 5	10 \pm 6	9 \pm 7	0.229
% Th2 cells	1.88 \pm 1.73	2.26 \pm 3.22	1.49 \pm 1.57	1.38 \pm 0.87	1.59 \pm 1.62	0.188
Number of Th17 cells/ μ L	7 \pm 4	7 \pm 4	7 \pm 5	8 \pm 4	6 \pm 4	0.524
% Th17 cells	1.80 \pm 3.21	2.32 \pm 3.98	1.19 \pm 0.75	1.15 \pm 0.48	1.26 \pm 1.45	0.144
Number of Treg cells/ μ L	29 \pm 13	27 \pm 14	34 \pm 16	35 \pm 12	36 \pm 16	0.032
% Treg cells	5.81 \pm 6.91	8.15 \pm 10.90	6.17 \pm 5.82	6.00 \pm 4.91	7.05 \pm 8.97	0.240
Th1/Th2 cell ratio	15.02 \pm 13.46	14.18 \pm 11.04	14.75 \pm 15.54	22.82 \pm 34.74	21.16 \pm 15.11	0.214
Th17/Treg cell ratio	0.29 \pm 0.19	0.28 \pm 0.18	0.23 \pm 0.16	0.26 \pm 0.15	0.19 \pm 0.14	0.014

Th cells: Helper T cells, NK cells: natural killer cells.

of CD4+ T cells, absolute numbers of CD8+ T cells and CD4+/CD8+ T cell ratios were observed among the groups ($P < 0.01$). We also identified significant differences in absolute numbers of Treg cells and Th17/Treg cell ratios ($P < 0.05$) between the different age groups.

To further assess relationships between age and lymphocyte subset composition, the data were analyzed using Spearman's correlation coefficients. Frequencies of CD4+ T cells ($r = 0.248$, $P < 0.001$) and CD4+/CD8+ T cell ratios ($r = 0.218$, $P = 0.002$) were positively correlated with advancing age. By contrast, absolute numbers of T cells ($r = -0.182$, $P = 0.011$), CD8+ T cells ($r = -0.211$, $P = 0.003$) and T+B+NK cells ($r = -0.178$, $P = 0.013$) were negatively correlated with advancing age. Among CD4+ T cell subsets, numbers of Th1 cells ($r = 0.147$, $P = 0.04$) and Treg cells ($r = 0.193$, $P = 0.007$) were positively correlated with advancing age. Conversely, the proportion of Th2 cells declined with age ($r = -0.172$, $P = 0.016$). Consequently, Th1/Th2 cell ratios ($r = 0.160$, $P = 0.025$) increased with age, and the Th17/Treg cell ratio ($r = -0.206$, $P = 0.004$) decreased with age. No other statistically significant correlations were identified (see Appendix).

Race-related differences in lymphocytes and CD4+ T cell subsets

Because reference intervals for lymphocytes and CD4+ T cell subsets may differ by race, we compared lymphocyte and CD4+ T cell subset reference ranges in diverse racial groups (Table 4⁹⁻¹³ and Table 5¹⁴). Absolute numbers of CD4+ T cells in Han Chinese individuals of the Shanxi region were lower than those in individuals from Hong Kong,⁹ Beijing,¹⁰ Germany¹² and Singapore.¹³ The CD4+/CD8+ ratios of Han Chinese individuals of the Shanxi region were similar to those reported in individuals from Beijing¹⁰ and Chongqing¹¹ but

differed from those determined in other districts or countries. The frequencies and absolute numbers of B cells were slightly lower in Han Chinese individuals of the Shanxi region than in individuals from Hong Kong,⁹ Germany¹² and Singapore.¹³ Compared with individuals in Italy,¹⁴ higher percentages and absolute numbers of Treg cells and lower percentages and absolute numbers of Th17 cells were observed in Han Chinese individuals of the Shanxi region.

Discussion

Variation in environmental factors and genetic inheritance can have major effects on standard reference intervals and immunity. To accurately report laboratory test results,¹⁵⁻¹⁷ it is necessary to establish sex-, age- and race-specific reference intervals for healthy individuals living in a particular region. In this study, we determined reference ranges of peripheral blood lymphocyte subpopulations and CD4+ T cell subsets in healthy Han Chinese adults in Shanxi Province, North China.

A close connection between different races and lymphocyte subsets has been clearly established. Studies of European and Asian individuals⁹⁻¹³ showed that the frequencies and absolute numbers of T cells, B cells and NK cells differed in some aspects. Compared the data reported in Italy,¹⁴ the percentages and absolute numbers of Treg cells were higher and those of Th17 cells were lower in Han Chinese individuals of the Shanxi region. These findings confirmed that lymphocyte subset composition was distinct across districts and countries. Generally speaking, lymphocyte subgroup reference ranges were distinct among different districts and races, even in different geographical regions of the same country.¹⁸ Laboratory test results and immune status may be influenced by subclinical infections, stress, and nutritional status in specific populations.¹⁹ This further

Table 4. Comparison of lymphocyte subset reference ranges in different ethnic groups.⁹⁻¹³

Parameter	Shanxi	Chongqing ¹¹	Hong Kong ⁹	Beijing ¹⁰	Singapore ¹³	Germany ¹²
Number of T cells/ μ L	746-2070	762-2043	723-2271	711-2353	796-2679	780-2240
% T cells	56.57-83	52.86-79.90	56.09-84.32	43.7-80.5	49-80	53-83
Number of B cells/ μ L	78-429	69-408	118-645	74-534	133-714	80-490
% B cells	5.00-20.28	4.50-18.10	7.19-25.85	4.4-21.2	7.0-28.0	5-21
Number of CD4+ T cells/ μ L	359-1122	384-1107	396-1309	368-1632	401-1451	490-1640
% CD4+ T cells	22.93-52.05	23.73-50.37	28.06-53.59	22.5-55.1	23-48.2	30-59
Number of CD8+ T cells/ μ L	193-976	198-867	224-1014	201-931	243-1206	170-880
% CD8+ T cells	12.93-46.19	13.73-38.64	16.37-42.65	11.2-43.1	13.4-41	10-40
CD4+/CD8+ T cell ratio	0.64-3.71	0.68-3.26	0.71-2.82	0.63-3.49	0.69-2.83	0.9-5.0
Number of NK cells/ μ L	101-708	78-697	61-607	63-1013	115-1009	80-690
% NK cells	5.80-31.05	6.17-32.67	3.66-26.74	3.7-46.1	6.0-37.0	5-32

NK cells: natural killer cells.

Table 5. Comparison of CD4⁺ T cell subset reference ranges in different ethnic groups.

Parameter	Shanxi	Italy ¹⁴
Number of Th1 cells/ μ L	14-275	144.20-171.40
% Th1 cells	4.85-162.21	6.66-8.01
Number of Th2 cells/ μ L	1-26	106.40-129.90
% Th2 cells	0.36-9.03	4.86-5.98
Number of Th17 cells/ μ L	1-18	201.3-242.00
% Th17 cells	0.32-8.10	9.50-11.47
Number of Treg cells/ μ L	5-64	1.79-17.41
% Treg cells	2.06-39.65	0.59-0.79
Th1/Th2 cell ratio	2.53-60.5	1.33-1.54
Th17/Treg cell ratio	0.06-0.70	0.06-0.11

Th cells: Helper T cells.

reinforces the importance of establishing local reference ranges to assist clinicians in making meaningful decisions.

The influence of sex on lymphocyte subgroups and CD4⁺ T cell subpopulations was also analyzed in this study. The absolute number of NK cells was lower in females than in males, in agreement with previous reports.^{8,9} Compared with women, CD4⁺/CD8⁺ T cell ratios were lower in men, consistent with results reported in Korean,⁸ Nigerian,²⁰ Ugandan,²¹ and South Floridian populations.¹⁶ Differences in the percentages of CD4⁺ T cells were unclear in previous studies. Some studies found no differences

between the sexes,^{9,16} while others suggested that the percentage of CD4⁺ T cells was generally low in males,^{8,21} in agreement with our study. Our results suggested that the proportion of Th1 cells was significantly lower in woman than in men, but that the absolute number of Th2 cells was increased in women (Table 2). These results contrasted with those of a previous study, which showed no differences in Th cells associated with gender.¹⁴ Different levels of sex hormones may underlie gender differences in lymphocyte subsets. For instance, levels of estrogen were higher in females and estrogen can block early T cell development in the thymus.²²

In this study, we identified relationships between lymphocyte subgroups and age. A positive correlation was identified between the proportion of CD4+ T cells and advancing age, while the absolute numbers of T cells and CD8+ T cells were inversely correlated with advancing age. Consequently, CD4+/CD8+ T cell ratios increased with age, in agreement with studies conducted in Beijing and Germany.^{10,12} Among CD4+ T cells, the numbers of Th1 and Treg cells were increased with age, and as a consequence Th17/Treg cell ratios decreased with age. Significant differences in the numbers of Treg and in Th17/Treg ratios were observed between age groups. However, there were no significant differences observed in another study.¹⁴ The effects of age on lymphocyte subsets and CD4+ T cell subsets are not completely understood. Decreases of T lymphocyte numbers, caused by thymic involution and potential changes in the activity of hematopoietic stem cells, were associated with aging.^{23,24} In addition, decreased function of the adaptive immune system was associated with aging, which may cause decreased CD8+ T cell numbers with advancing age.²⁵ Differences in lymphocyte composition among age groups suggested that it was necessary to establish age-specific reference intervals for lymphocytes and CD4+ T cell subsets to accurately interpret laboratory results.

Our study had several limitations. Changes in reference intervals might be due to technical or methodological reasons. For instance, single-platform and dual platform detection methods show different diagnostic parameters. The National Institutes of Health recommends using a single platform instead of the dual platform counting method to reduce differences between different laboratories and to produce more reliable data.²⁶

Conclusion

We used single-platform flow cytometry to establish reference ranges for lymphocytes

and CD4+ T cell subsets among healthy Han Chinese individuals of the Shanxi region. We compared these ranges with the results of previous studies. Differences in the composition of lymphocyte and CD4+ T subpopulations were associated with age, sex and race. Therefore, to aid clinical decision-making, we must establish reference intervals specific to patient subgroups. The results of our study might help to understand illness- or treatment-related changes in lymphocyte populations in the context of transplantation, cancer, infection, autoimmunity and immunodeficiency.

Abbreviations

Th: T helper cells; Ts: suppressor T cells; Treg: regulatory T cells; NK cells: natural killer cells; SD: standard deviation; FITC: fluorescein isothiocyanate; PE: phycoerythrin; APC: allophycocyanin; PerCP: peridinin-chlorophyll protein; EDTA: ethylenediaminetetraacetic acid

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Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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References

1. Noack M and Miossec P. Th17 and regulatory T cell balance in autoimmune and inflammatory diseases. *Autoimmun Rev* 2014; 13: 668–677.
2. Morita R, Schmitt N, Bentebibel SE, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* 2011; 34: 108–121.
3. Sallusto F. Heterogeneity of human CD4(+) T cells against microbes. *Annu Rev Immunol* 2016; 34: 317–334.
4. Messemaker TC, Huizinga TW and Kurreeman F. Immunogenetics of rheumatoid arthritis: understanding functional implications. *J Autoimmun* 2015; 64: 74–81.
5. Lee SE, Yahng SA, Cho BS, et al. Lymphocyte subset analysis for the assessment of treatment-related complications after autologous stem cell transplantation in multiple myeloma. *Cytotherapy* 2012; 14: 505–512.
6. Thompson MA, Aberg JA, Cahn P, et al. Antiretroviral treatment of adult HIV infection: 2010 recommendations of the International AIDS Society-USA panel. *JAMA* 2012; 304: 321–333.
7. Jalla S, Sazawal S, Deb S, et al. Enumeration of lymphocyte subsets using flow cytometry: effect of storage before and after staining in a developing country setting. *Indian J Clin Biochem* 2004; 19: 95–99.
8. Choi J, Lee SJ, Lee YA, et al. Reference values for peripheral blood lymphocyte subsets in a healthy Korean population. *Immune Netw* 2014; 14: 289–295.
9. Wong WS, Lo AWI, Siu LP, et al. Reference ranges for lymphocyte subsets among healthy Hong Kong Chinese adults by single-platform flow cytometry. *Clin Vaccine Immunol* 2013; 20: 602–606.
10. Jiao Y, Qiu Z, Xie J, et al. Reference ranges and age-related changes of peripheral blood lymphocyte subsets in Chinese healthy adults. *Sci China C Life Sci* 2009; 52: 643–650.
11. Zhang K, Wang F, Zhang M, et al. Reference ranges of lymphocyte subsets balanced for age and gender from a population of healthy adults in Chongqing District of China. *Cytometry B Clin Cytom* 2016; 90: 538–542.
12. Jentsch-Ullrich K, Koenigsmann M, Mohren M, et al. Lymphocyte subsets' reference ranges in an age- and gender-balanced population of 100 healthy adults—a monocentric German study. *Clin Immunol* 2005; 116: 192–197.
13. Chng WJ, Tan GB and Kuperan P. Establishment of adult peripheral blood lymphocyte subset reference range for an Asian population by single-platform flow cytometry: influence of age, sex, and race and comparison with other published studies. *Clin Diagn Lab Immunol* 2004; 11: 168–173.
14. Sorrenti V, Marena B, Fortinguerra S, et al. Reference values for a panel of cytokinergic and regulatory lymphocyte subpopulations. *Immune Netw* 2016; 16: 344–357.
15. Marcos A, Nova E and Montero A. Changes in the immune system are conditioned by nutrition. *Eur J Clin Nutr* 2003; 57: S66–S69.
16. Valiathan R, Deeb K, Diamante M, et al. Reference ranges of lymphocyte subsets in healthy adults and adolescents with special mention of T cell maturation subsets in adults of South Florida. *Immunobiology* 2014; 219: 487–496.
17. Hussain T, Abbas S, Khan MA, et al. Lysine fortification of wheat flour improves selected indices of the nutritional status of predominantly cereal-eating families in Pakistan. *Food Nutr Bull* 2004; 25: 114–122.
18. Torres AJ, Angelo AL, Netto EM, et al. Reference range for T lymphocytes populations in blood donors from two different regions in Brazil. *Braz J Infect Dis* 2009; 13: 221–225.
19. Al-Mawali A, Pinto AD, Al Busaidi R, et al. Lymphocyte subsets: reference ranges in an age- and gender-balanced population of Omani healthy adults. *Cytometry A* 2013; 83: 739–744.
20. Oladepo DK, Idigbe EO, Audu RA, et al. Establishment of reference values of CD4 and CD8 lymphocyte subsets in healthy

- Nigerian adults. *Clin Vaccine Immunol* 2009; 16: 1374–1377.
21. Tollerud DJ, Clark JW, Brown LM, et al. The influence of age, race, and gender on peripheral blood mononuclear-cell subsets in healthy nonsmokers. *J Clin Immunol* 1989; 9: 214–222.
 22. Rijhsinghani AG, Thompson K, Bhatia SK, et al. Estrogen blocks early T cell development in the thymus. *Am J Reprod Immunol* 1996; 36: 269–277.
 23. Linton PJ and Dorshkind K. Age-related changes in lymphocyte development and function. *Nat Immunol* 2004; 5: 133–139.
 24. Crooke SN, Ovsyannikova IG, Poland GA, et al. Immunosenescence and human vaccine immune responses. *Immun Ageing* 2019; 16: 25.
 25. Weng NP. Aging of the immune system: how much can the adaptive immune system adapt? *Immunity* 2006; 24: 495–499.
 26. Bergeron M, Faucher S, Ding T, et al. Evaluation of a universal template for single-platform absolute T-lymphocyte subset enumeration. *Cytometry* 2002; 50: 62–68.

Appendix. Correlations between age and absolute counts and percentages of lymphocyte subsets

Parameter	Correlation coefficient (r)	P-value
Number of T cells/ μ L	-0.182	0.011
% T cells	0.038	0.6
Number of B cells/ μ L	-0.1	0.164
% B cells	-0.016	0.826
Number of CD4+ T cells/ μ L	0.03	0.673
% CD4+ T cells	0.248	<0.001
Number of CD8+ T cells/ μ L	-0.211	0.003
% CD8+ T cells	-0.112	0.117
CD4+/CD8+ T cell ratio	0.218	0.002
Number of NK cells/ μ L	-0.122	0.088
% NK cells	-0.021	0.771
T+B+NK cells/ μ L	-0.178	0.013
% T+B+NK cells	0.139	0.051
Number of Th1 cells/ μ L	0.147	0.04
% Th1 cells	0.077	0.284
Number of Th2 cells/ μ L	-0.058	0.423
% Th2 cells	-0.172	0.016
Number of Th17 cells/ μ L	0.001	0.987
% Th17 cells	-0.103	0.150
Number of Treg cells/ μ L	0.193	0.007
% Treg cells	0.134	0.062
Th1/Th2 cell ratio	0.160	0.025
Th17/Treg cell ratio	-0.206	0.004

Th cells: Helper T cells, NK cells: natural killer cells