



Research Article

Reference range of naïve T and T memory lymphocyte subsets in peripheral blood of healthy adult

Ying Xia^{1,2,4,†}, Aqing Liu^{1,2,4,†}, Wentao Li^{1,†}, Yunhe Liu¹, Guan Zhang^{1,2,4}, Songshan Ye^{1,2,4}, Zhijieruo Zhao^{1,2,4}, Juan Shi¹, Yingjie Jia¹, Xu Liu³, Yongtie Guo³, Huayu Chen³, and Jianchun Yu^{1,*} 

¹Oncology Department, First Teaching Hospital of Tianjin University of Traditional Chinese Medicine, Tianjin, China

²Graduate School, Tianjin University of Traditional Chinese Medicine, Tianjin, China

³Clinic Laboratory, First Teaching Hospital of Tianjin University of Traditional Chinese Medicine, Tianjin, China

⁴National Clinical Research Center for Chinese Medicine Acupuncture and Moxibustion, Tianjin, China

*Correspondence: Jianchun Yu, Oncology Department, First Teaching Hospital of Tianjin University of Traditional Chinese Medicine, Tianjin 300193, China.
E-mail: yujianchun2000@163.com

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

Abstract

Naïve T and T memory cell subsets are closely related to immune response and can provide important information for the diagnosis and treatment of immunological and hematological disorders. Lymphocyte compartment undergoes dramatic changes during adulthood; age-related reference values derived from healthy individuals are crucial. However, extensively detailed reference values of peripheral blood lymphocytes in the whole spectrum of adulthood detected by multi-color flow cytometry on a single platform are rare. Three hundred and nine healthy adult volunteers were recruited from Tianjin in China. The absolute counts and percentages of CD3⁺CD4⁺ T cells, CD3⁺CD8⁺ T cells, naïve T cells (Tn), T memory stem cells (Tscm), central memory T cells (Tcm), effector memory T cells (Tem), and terminal effector T cells (Tte) were detected by flow cytometry with single platform technologies. Reference range of absolute counts and percentage of T lymphocyte subsets were formulated by different age and gender. The results showed that Tn and Tscm cells, which had stem cell properties, decreased with aging; while, Tcm and Tem increased with aging, which increased from 18 to 64 years old but presented no significant change over the 65 years old. Gender had an influence on the fluctuation of lymphocyte subsets, the absolute count of CD3⁺CD8⁺, CD8⁺Tcm, CD8⁺Tem in males were higher than those in females. The reference values of percentages and absolute numbers of naïve T and T memory cell subsets can help doctors to understand the immune state of patients and evaluate conditions of prognosis then adjust the treatment for patients. (Chinese Clinic Trial Registry number: ChiCTR-IOR-17014139.)

Keywords: naïve T cell, T memory cell subsets, reference range, changing regularity, flow cytometry

Abbreviations: Tn, naïve T cells; Tm, T memory cells; Tscm, T memory stem cells; Tcm, central memory T cells; Tem, effector memory T cells; Tte, terminal effector T cells; M. tb, *Mycobacterium tuberculosis*

Introduction

Long-lived memory T cells maintained long-time immunological memory in the host [1, 2]. The immune system gained a long-term ability of responding to a broad diverse spectrum of pathogens and tumor cell antigens through developing special lymphocyte differentiation programs to ensure the duration of a given antigen-specific immune response [3, 4]. Upon antigen stimulation, Tn entered distinct cell programs of development and differentiation and then produced Tscm, Tcm, and Tem [5]. The key mediator in this process was Tscm, a kind of multipotent progenitor that owned the capacity of self-renewing and could replenish more differentiated subsets of memory T cells, including Tcm, Tem, and Tte [6]. Tscm played a vital role in many physiological and pathological human processes owing to their extreme longevity and robust potential for immune reconstitution [7]. Some studies

indicated that CD4⁺CD45RA⁺CD95⁺T cells from patients with lung cancer exhibiting stronger anti-tumor function possessed certain memory cell phenotypes which could help contribute to favorable prognostic factors of disease [8]. In infection disease, such as infected with *Mycobacterium tuberculosis* (M. tb), M. tb-specific Tscm would be produced in the host, which was also functional and could produce IL-2, IFN- γ , TNF- α upon antigen stimulation, and the percentages of Tscm were correlated positively with long-term Calmette-Guerin-specific CD4⁺ T cell proliferative potential after infant vaccination [9].

Detecting the changes of the circulating T lymphocyte subsets can be beneficial to monitor the onset and progression of the disease and determine optimal treatment [10]. Therefore, establishing a reference range for Tn and Tm lymphocyte subsets is crucial for analyzing the clinical status of the immune

and its response. But there was a rare reference range of naïve T and T memory cell subsets provided by the single platform method, the need of precision reference range of naïve T and T memory cell subsets at different age stages prompted us to carry out this work. Flow cytometric analysis is a convenient and efficient method for studying immune status and has been widely used in clinical diagnosis and administrating of immune diseases associated with phenotypic and functional perturbations of lymphocyte subsets [11].

So far, almost all absolute numbers of lymphocyte subsets in the peripheral circulation have been detected by dual-platform technique in a traditional way which couples percentages of positive cell subsets determined by flow cytometry with the absolute lymphocyte count obtained by automated hematology analyzers [10, 12]. It was indicated that this conventional, universal technique was responsible for substantial differences in absolute lymphocyte counts reported by different laboratories [13, 14]. The more advanced method of a single platform, which was performed completely on the flow cytometer, had effectively increased the assay precision and allowed for greater uniformity of results between laboratories [13–15]. So, the percentages and absolute numbers of these subsets were tested by ten-color flow cytometer based on a single-platform technique.

We examined Tn and Tm subsets of 309 healthy volunteers, some regional data of lymphocyte phenotypes showed

variations due to the influence of gender, age, ethnicity, and lifestyle differences [16], gender and age were the most common influence factors, therefore, we explored reference ranges based on age and gender.

Materials and methods

Clinical data

All the subjects were given informed consent in accordance with the Declaration of Helsinki and the clinical trial was approved by the hospital ethics committee (TYLL2017[K]002) and registered at the Chinese Clinic Trial Registry (ChiCTR-IOR-17014139). A total of 309 healthy adult (171males and 138 females) volunteers ranging from 18 to 88 years old were recruited from Tianjin in China between 1 September 2019 and 1 July 2020. According to the aging definition by the National Health and Family Planning Commission of the People's Republic of China, the subjects were classified into three age groups as follows: 18–44 years ($n = 101$); 45–65years ($n = 106$) and >65years ($n = 102$) [10].

Inclusion criteria

All the subjects were healthy without diseases related to abnormal of heart, brain, liver, kidney, hematology, immune system, and so on. Physical examination, blood routine

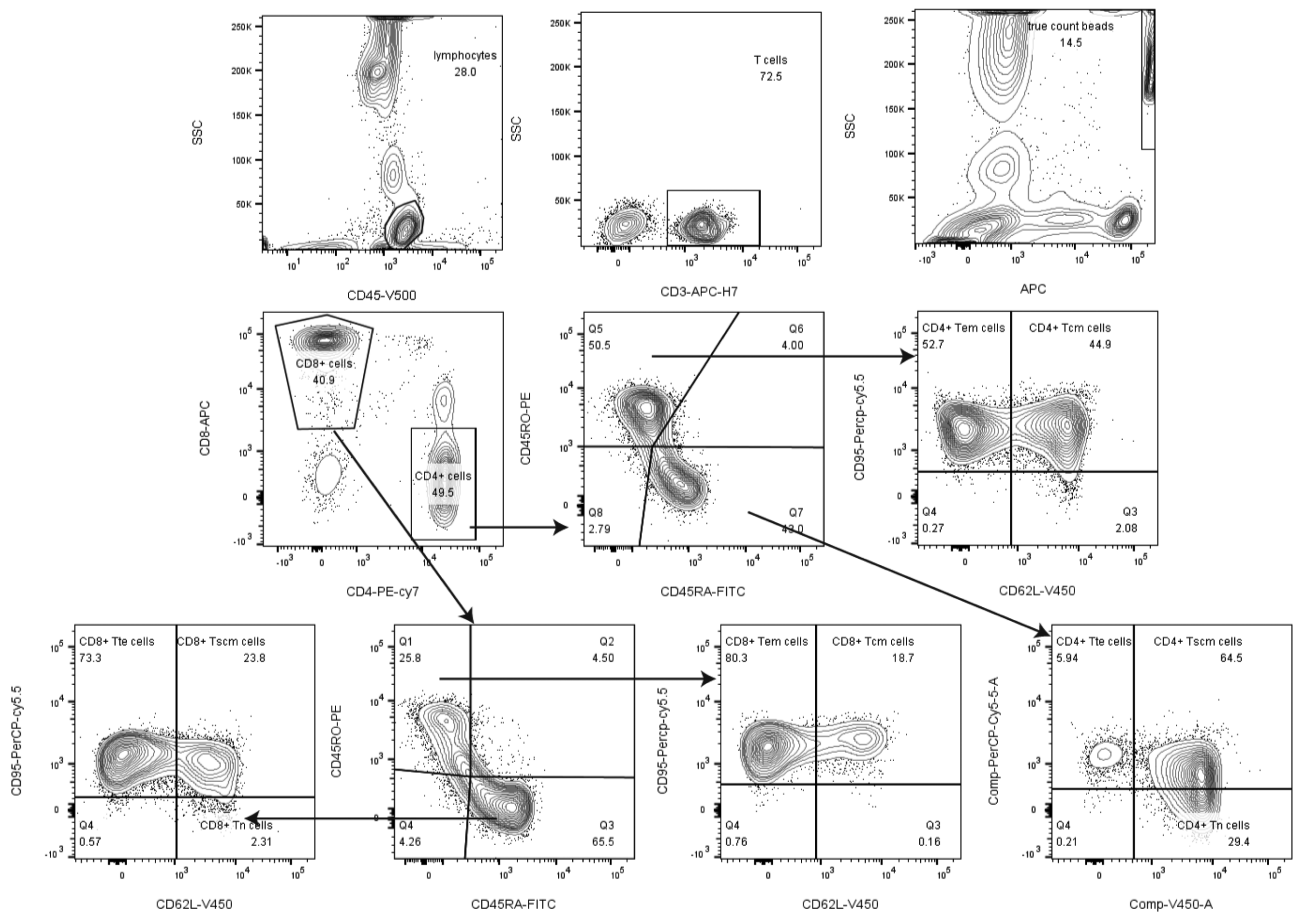


Fig. 1 Gating strategies. Firstly, we gated lymphocyte identified by CD45 from leukocyte, then gated CD3+ T cells from lymphocyte. T cell subsets populations Tcm (CD95+ CD62L-) and Tem (CD95+ CD62L+) were gated from CD3+CD4+ (CD45RO+ CD45RA-) and CD3+CD8+ (CD45RO+ CD45RA-)T subsets; Tscm (CD95+CD62L+), Tn (CD95-CD62L+) and Tte (CD95+CD62L-) were gated from CD3+CD4+ (CD45RO-CD45RA+) and CD3+CD8+ (CD45RO-CD45RA+)T subsets.

examination, liver functions, renal functions, and blood glucose levels were normal.

Exclusion criteria

Except for the diseases including influenza, systemic infection, autoimmune diseases, connective tissue disease, HIV, abnormal tumor marker, or cancer that caused the abnormal of immune.

Reagents and instruments

The T lymphocyte subsets were analyzed using a lyse/no-wash procedure based on a single-platform technique by ten-color flow cytometry (BD FACS Canto II:U657338000541). The main reagents were Percp-cy-labeled mouse Anti-Human CD95 (cat: 561655), Bv421-labeled mouse Anti-Human CD62L (cat: 563862), PE-cy7-labeled mouse Anti-Human CD4 (cat: 663493), APC-labeled mouse Anti-Human CD8 (cat: 663524), APC-H7-labeled mouse Anti-Human CD3 (cat: 663490), V-500-C labeled mouse Anti-Human CD45 (cat: 662912), PE-labeled mouse Anti-Human CD45RO (cat: 663530), FITC-labeled mouse Anti-Human CD45RA (cat: 662840), BD Multitest hemolysin (340503). The EDTA blood collecting tubes and trucount tubes (340334) were also from BD Biosciences.

Sample collection

Two milliliters of EDTA anticoagulated fresh peripheral blood were obtained from healthy adults, intensively mixed by turning upside-down immediately.

Cellular staining and analysis

The procedure was performed by the flow cytometer with single platform technique. The manipulation was done according to BD operating instruction. CD3+CD4+T cells, CD3+CD8+T cells, Tn, Tscm, Tcm, Tem, and Tte were identified according to published protocols, such as Tn(CD45RA+CD45RO-CD62L+CD95-), Tscm(CD45RA+CD45RO-CD62L+CD95+), Tcm(CD45RA-CD45RO+CD62L+CD95+), Tem(CD45RA-CD45RO+CD62L-CD95+), Tte(CD45RA+CD45RO-CD62L-CD95+) [7, 17–19].

- (1) For each sample, 20 μ l of PE-labelled mouse Anti-Human CD45RO and FITC-labelled mouse Anti-Human CD45RA reagents were respectively pipetted into the bottom of trucount tubes; then 5 μ l of Percp-cy-labelled mouse Anti-Human CD95, Bv421-labelled mouse Anti-Human CD62L, PE-cy7-labelled mouse Anti-Human CD4, APC-labelled mouse Anti-Human CD8, APC-H7-labelled mouse Anti-Human CD3, and V-500-C-labelled

Table 1. Reference range of absolute counts of T lymphocyte subsets in different age groups

Parameters		All	(18–44) years	(45–65) years	>65 years
		n = 309	n = 101	n = 106	n = 102
Age	Mean \pm SD	50.52 \pm 18.00	30.73 \pm 6.52	54.63 \pm 6.37	73.59 \pm 6.60
Sex	Male:female	171:138	56:45	56:50	59:43
Lymphocyte counts (cells/ μ l)	Median	1766.39	1692.59	1791.59	1668.16
	Reference range <i>CI</i> 90%	(1201.93–2509.45)	(1154.13–2222.62)	(1289.37–2569.88)	(1089.05–2858.61)
CD3+ (cells/ μ l)	Median	1139.49	1137.67	1203.23	1045.59
	Reference range <i>CI</i> 90%	(744.15–1680.36)	(735.08–1581.36)	(750.31–1717.94)	(721.92–1791.96)
CD3+CD4+ (cell/ μ l)	Median	585.91	538.20	647.07	562.21
	Reference range <i>CI</i> 90%	(364.51–960.9)	(361.84–908.7)	(360.52–964.2)	(365.22–1052.91)
CD4+Tn (cells/ μ l)	Median	25.11	34.38	23.06	14.9
	Reference range <i>CI</i> 90%	(5.05–76.43)	(10.56–74.91)	(5.13–87.66)	(2.57–57.36)
CD4+Tscm (cells/ μ l)	Median	102.90	140.9	85.49	69.2
	Reference range <i>CI</i> 90%	(19.94–306.54)	(37.85–333.11)	(15.10–281.59)	(9.41–280.89)
CD4+Tcm (cells/ μ l)	Median	154.85	141.74	183.84	155.81
	Reference range <i>CI</i> 90%	(76.43–323.05)	(70.26–223.04)	(86.00–343.58)	(77.75–401.63)
CD4+Tem (cells/ μ l)	Median	116.59	100.20	132.72	123.77
	Reference range <i>CI</i> 90%	(58.07–268.76)	(54.47–160.63)	(65.23–272.08)	(57.17–336.65)
CD4+Tte (cells/ μ l)	Median	2.41	2.24	2.58	2.94
	Reference range <i>CI</i> 90%	(0.20–50.52)	(0.35–53.06)	(0.17–47.15)	(0.14–58.93)
CD3+CD8+ (cell/ μ l)	Median	423.88	449.64	404.97	323.01
	Reference range <i>CI</i> 90%	(210.24–705.0)	(224.66–669.0)	(212.49–700.9)	(181.96–892.3)
CD8+Tn (cells/ μ l)	Median	12.42	27.5	10.49	3.01
	Reference range <i>CI</i> 90%	(1.31–46.06)	(5.78–51.99)	(2.18–31.37)	(0.58–17.08)
CD8+Tscm (cells/ μ l)	Median	57.24	110.47	48.63	18.67
	Reference range <i>CI</i> 90%	(6.10–212.35)	(30.89–261.21)	(11.21–109.95)	(3.39–99.07)
CD8+Tcm (cells/ μ l)	Median	36.15	31.18	36.70	37.61
	Reference range <i>CI</i> 90%	(12.74–96.56)	(11.49–70.20)	(13.10–102.61)	(12.59–129.56)
CD8+Tem (cells/ μ l)	Median	85.75	94.33	83.44	75.19
	Reference range <i>CI</i> 90%	(24.34–247.25)	(21.45–209.90)	(26.51–222.79)	(19.26–286.57)
CD8+Tte (cells/ μ l)	Median	53.23	47.93	57.55	38.05
	Reference range <i>CI</i> 90%	(6.79–181.57)	(6.97–136.30)	(4.52–241.49)	(4.97–283.41)

- mouse Anti-Human CD45 were added into the bottom of trucount tubes respectively
- (2) Next, 50µl of well-mixed and anti-coagulated whole blood was pipetted into the bottom of every tube.
 - (3) Then vortexed gently to mix, incubated for 15 min in dark at room temperature.
 - (4) Finally, 400µl of 1×BD Multitest IMK kit lysing solution was pipetted into every tube. The solution was vortexed gently to mix and incubated for 15 min in dark at room temperature.
 - (5) Samples were analyzed on the flow cytometer.

Statistical analysis

Statistical analysis was performed by SPSS software 25.0. Kolmogorov–Smirnov was used for the distribution test. Reference ranges were presented as the median together with the 5th and 95th percentile (90% confidence interval). Comparisons among three variables were performed using the Kruskal–Wallis test. Variables were grouped by gender

comparing with Mann–Whitney *U* test for non-parametric data. Using a non-parametric Spearman's rank correlation test to analyse the association between variables and age. Probability value was obtained from two-sided tests and *P* < 0.05 was considered statistically significant.

Result

Gating strategy

The gating strategy for naïve T and T memory cell subsets is shown in Fig. 1. Firstly, we gated lymphocyte identified by CD45 from leukocyte, then gated CD3+ T cells from lymphocyte. Tcm (CD95+ CD62L-) and Tem (CD95+CD62L+) were gated from CD3+CD4+ (CD45RO+ CD45RA-) and CD3+CD8+ (CD45RO+ CD45RA-) T subsets; Tscm (CD95+ CD62L+), Tn (CD95-CD62L+) and Tte (CD95+CD62L-) were gated from CD3+CD4+ (CD45RO-CD45RA+) and CD3+CD8+ (CD45RO-CD45RA+) T subsets.

Table 2. Reference range of percentages of T lymphocyte subsets in different age groups

Parameters		All	(18–44) years	(45–65) years	>65 years
		<i>n</i> = 309	<i>n</i> = 101	<i>n</i> = 106	<i>n</i> = 102
Age	Mean±SD	50.52 ± 18.00	30.73 ± 6.52	54.63 ± 6.37	73.59 ± 6.60
Sex	Male:female	171:138	56:45	56:50	59:43
CD3+%lymphocyte cells	Median	67.90%	68.5%	66.55%	64.2%
	Reference range <i>CI</i> 90%	(49.58–79%)	(55.33–79.28%)	(50.06–78.53%)	(48.52%–81.26%)
CD3+CD4+%CD3+	Median	52.8%	50.4%	53.95%	57.05%
	Reference range <i>CI</i> 90%	(37%–68.34)	(34.6–65.46%)	(41.85%–69.49%)	(35.43%–71.17%)
CD3+CD4+%lymphocyte cells	Median	34.1%	32.35%	35.05%	35.1%
	Reference range <i>CI</i> 90%	(23.86–47.86%)	(23.7–45.81%)	(25.64–48.49%)	(19.36–50.13%)
CD4+Tn%CD3+CD4+	Median	4.3%	6%	3.45%	2.6%
	Reference range <i>CI</i> 90%	(1–10.82%)	(2.14–11.62%)	(1.1–11.16%)	(0.52–7.09%)
CD4+Tscm%CD3+CD4+	Median	17.6	26.35	13.55	11.4
	Reference range <i>CI</i> 90%	(3.3–38.94%)	(8.34–41.30%)	(3.3–35.77%)	(2.02–30.55%)
CD4+Tcm%CD3+CD4+	Median	27.00%	26.1%	28.1%	26.45%
	Reference range <i>CI</i> 90%	(14.7–43.42%)	(11.41–38.13%)	(16.37–43.20%)	(14.81–52.9%)
CD4+Tem%CD3+CD4+	Median	20.3%	17.6%	22.1%	20.85%
	Reference range <i>CI</i> 90%	(10.48–40.2%)	(8.03–34.67%)	(10.88–42.27%)	(10.27–42.41)
CD4+Tte%CD3+CD4+	Median	0.4%	0.5%	0.4%	0.7%
	Reference range <i>CI</i> 90%	(0.01–9.06%)	(0.01–10.24%)	(0.01–8.58%)	(0.01–10.7%)
CD3+CD8+%CD3+	Median	36.8%	38%	35.3%	34.8%
	Reference range <i>CI</i> 90%	(23–51.44%)	(25.13–53.70%)	(22.98–48.7%)	(18.25–57.71%)
CD3+CD8+%Lymphocyte cells	Median	24.9%	26.15%	22.85%	20.85%
	Reference range <i>CI</i> 90%	(13.1–37.42%)	(16.56–40.02%)	(13.38–35.17%)	(10.39–37.97%)
CD8+Tn%CD3+CD8+	Median	3.2%	6.45%	2.5%	0.8%
	Reference range <i>CI</i> 90%	(0.3–9.34%)	(2.05–10.33%)	(0.5–8.02%)	(0.11–4.75%)
CD8+Tscm%CD3+CD8+	Median	13.7%	28.05%	11.6%	6.1%
	Reference range <i>CI</i> 90%	(1.9–38.42%)	(8.30–48.90%)	(2.67–31.81%)	(1.3–21.86%)
CD8+Tcm%CD3+CD8+	Median	8.9%	7.4%	8.8%	12.15%
	Reference range <i>CI</i> 90%	(3.38–21.04%)	(2.73–15.05%)	(3.41–21.46%)	(4.91–27.45%)
CD8+Tem%CD3+CD8+	Median	22.8%	22.3%	21.35%	27.45%
	Reference range <i>CI</i> 90%	(6–48.8%)	(5.49–37.72%)	(6.94–49.84%)	(4.91–27.45%)
CD8+Tte%CD3+CD8+	Median	12.2%	10.5%	13.85%	10.6%
	Reference range <i>CI</i> 90%	(1.9–38.42%)	(2.00–30.62%)	(1.77–39.05%)	(1.47–45.85%)

Reference range of T lymphocyte subsets in different age groups

The healthy volunteers were recruited for assessment of human T lymphocyte subsets including 171 males (55.33 %) and 138 females (44.67 %). Hundred and one (32.7%) were in 18–44 years group (56 males, 45 females, mean age 30.73 years), 106 (34.3%) belonged to 45–65 years group (56 males, 50 females, mean age 54.63 years) and 102 (33.0%) belonged to the over 65 years old group (59 males, 43 females, mean age 73.59 years). The Kolmogorov–Smirnov test demonstrated that absolute counts and percentages of each subsets were abnormal distribution among the three cohorts ($P < 0.001$). So we used the percentile method to determine the reference range of each parameter. The median and reference range of absolute counts and percentage for each group are shown in Table 1,2.

The difference of absolute counts of T lymphocyte subsets in each age group

To analyze the absolute counts further, the data in Table 1 were performed by statistics. The absolute counts of CD4+Tn and Tscm decreased gradually with aging among the three groups, the median of CD4+Tn were 34.38 cells/ μ l, 23.06 cells/ μ l, and 14.9 cells/ μ l in each group with aging, respectively ($P < 0.05$, Fig. 2A), and the median of CD4+Tscm were 140.9 cells/ μ l, 85.49 cells/ μ l, and 69.2 cells/ μ l in every group respectively ($P < 0.05$, Fig. 2B). The same changes could be

seen in CD8+Tn and CD8+Tscm cell populations ($P < 0.05$, Fig. 2F and G). The absolute counts of CD4+Tcm, CD4+Tem increased with aging ($P < 0.05$, Fig. 2C and D), but there was no obvious difference between the group of 45–64 years old and over 65 years old ($P > 0.05$, Fig. 2C and D). The absolute count of CD8+Tcm showed increased with aging which was also similar to CD4+Tcm ($P < 0.05$, Fig. 2H). The absolute counts of CD4+Tte, CD8+Tem, CD8+Tte did not change with aging ($P > 0.05$, Fig. 2E, I, and J).

Age-related T cell changes in distribution

There was a weak negative correlation between age and the counts of CD4+Tn ($r = -0.379$, $P < 0.01$, Fig. 3A) and CD4+Tscm ($r = -0.335$, $P < 0.01$, Fig. 3B) which stated that the numbers of CD4+Tn and CD4+Tscm did not fluctuate much. A strong negative correlation between age and the counts of CD8+Tn ($r = -0.718$, $P < 0.01$, Fig. 3E) and CD8+Tscm ($r = -0.656$, $P < 0.01$, Fig. 3F) suggested that the numbers of CD8+Tn and CD8+Tscm fluctuated significantly with aging. The weak positive correlation between age and the counts of CD4+Tcm ($r = 0.261$, $P < 0.01$, Fig. 3C), CD4+Tem ($r = 0.280$, $P < 0.01$, Fig. 3D), CD8+Tcm ($r = 0.171$, $P < 0.01$, Fig. 3G) were showed in our study. The cells which have stem cell properties, such as Tn, Tscm cells decreased with age. However, memory cell subsets, which have no stem cell properties such as Tcm, Tem cells increased with age (Fig. 3I and H).

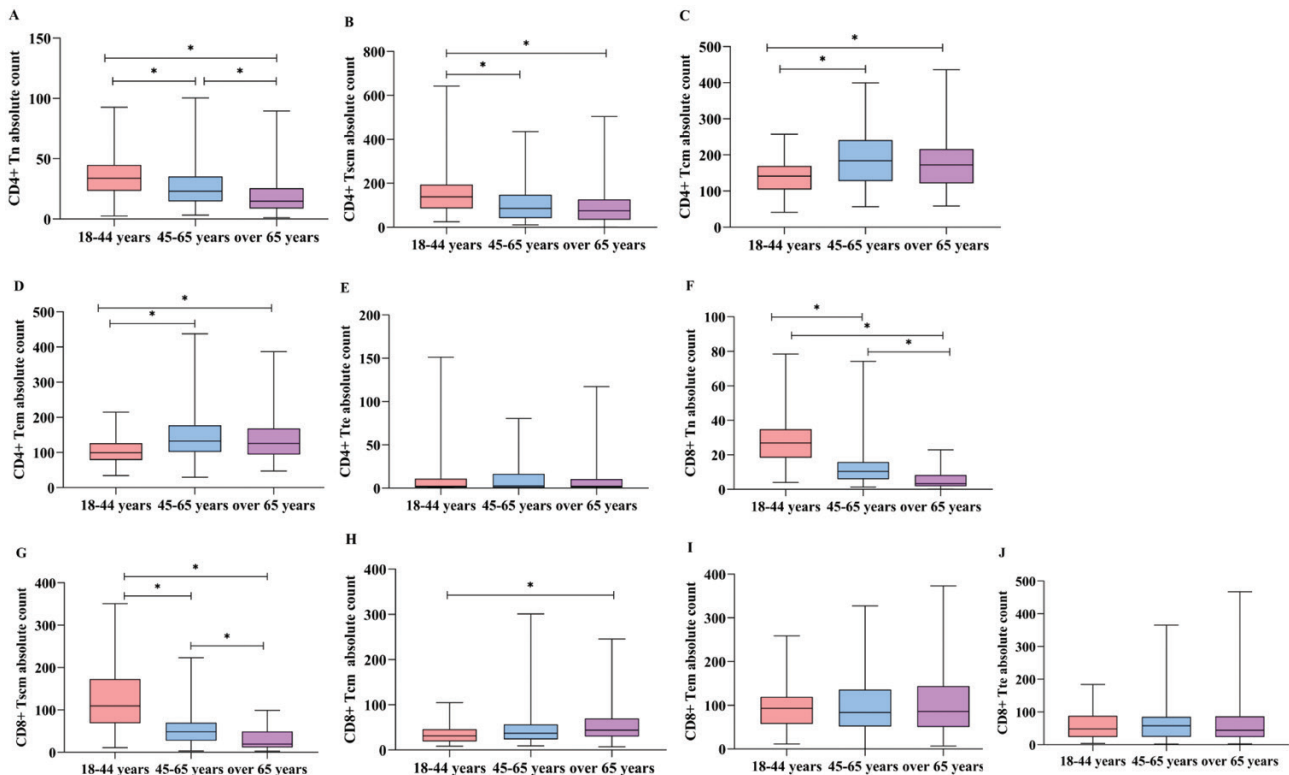


Fig. 2 Comparison of absolute counts of T lymphocyte subsets in different age groups. (A, B, F, G) showed the absolute count of CD4+Tn, CD4+Tscm, CD8+Tn, CD8+Tscm in group of 18–45 years were higher than those in group of 45–65years and over 65years ($P < 0.05$); (C, D) stated that the absolute counts of CD4+Tcm, CD4+Tem in groups of 45–64 years old and over 65 years old were higher than those in 18–45 years old ($P < 0.05$), but there were no difference between the group of 45–64 years old and over 65 years old ($P > 0.05$); (H) indicated that the absolute count of CD8+Tcm in the over 65 years old group was more than those in the 18–45 years old ($P < 0.05$); (E, I, J) demonstrated that the absolute counts of CD4+Tte, CD8+Tem, CD8+Tte did not changes with age (* represents significant differences).

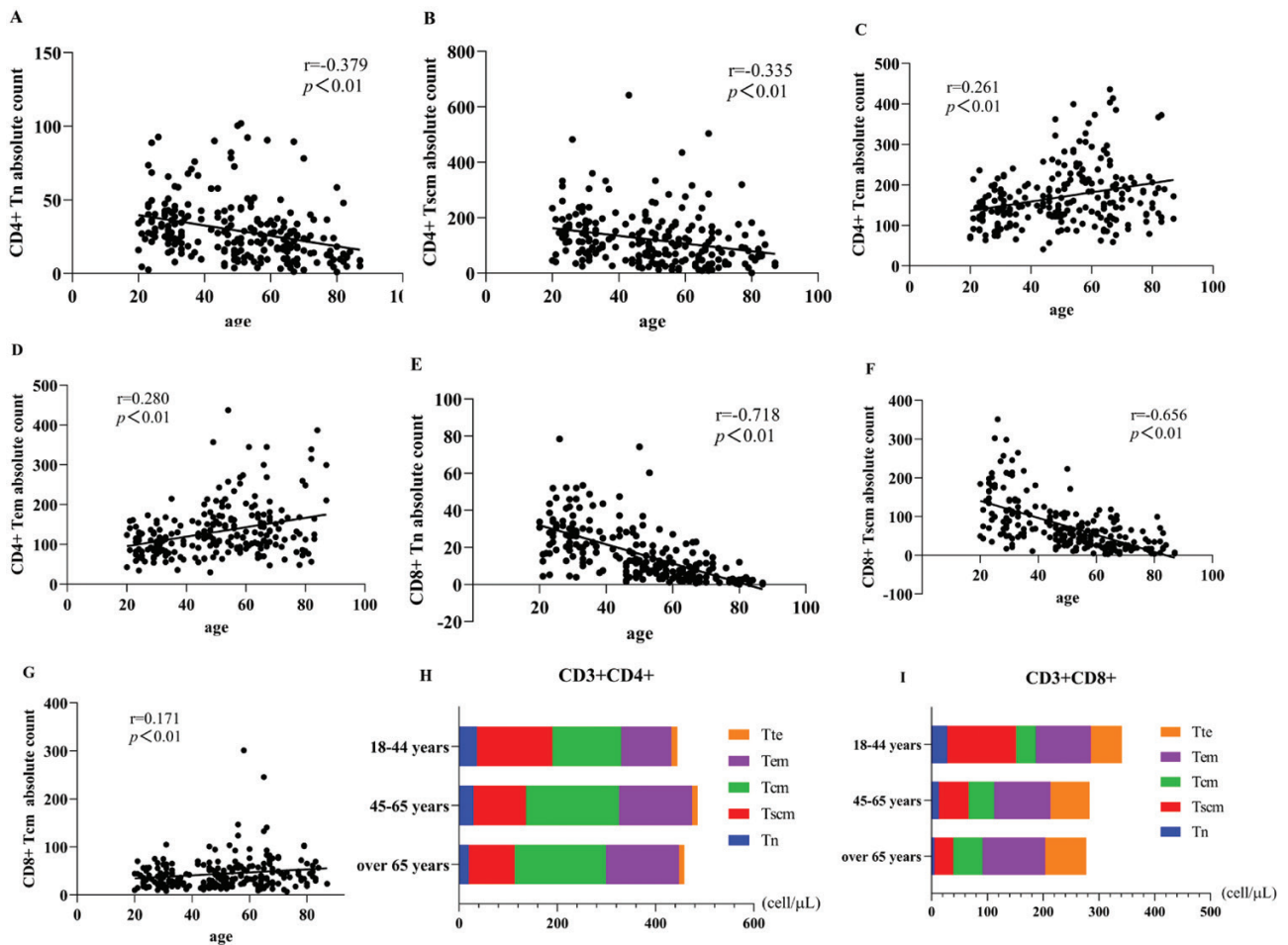


Fig. 3 Relationship between age and T lymphocyte subsets and changes in distribution. (A, B, E, F) showed a trend of decrease in CD4+Tn cell counts ($r = -0.379$, $P < 0.01$), CD4+Tscm cell counts ($r = -0.335$, $P < 0.01$), CD8+Tn cell counts ($r = -0.718$, $P < 0.01$), and CD8+ Tscm cell counts ($r = -0.656$, $P < 0.01$) with increased age. (C, D, G) indicated an increase trend with aging in CD4+Tcm ($r = 0.261$, $P < 0.01$), CD4+Tem ($r = 0.280$, $P < 0.01$), CD8+Tcm ($r = 0.171$, $P < 0.01$). (I, H) represented that the Tn, Tscm cells which had stem cell properties decreased with aging; however, memory cell subsets, such as Tcm, Tem increased with aging (* represents significant differences).

Reference range of T lymphocyte subsets in different gender

Gender has influences on the fluctuation of lymphocyte subsets [10]. So we established the reference range of lymphocyte subgroups according to gender (Tables 3 and 4). Compared parameters between genders, absolute counts of CD3+CD8+, CD8+Tcm, CD8+Tem in the male group were higher than those in the female group ($P < 0.05$, Fig. 4A-C).

The difference of Tte in CD4+ and CD8+ cells

The analysis showed that the absolute counts of CD4+Tte and CD8+Tte were different, the absolute counts of Tte in CD8+ were higher than that in CD4+ ($P < 0.05$, Fig. 5).

Discussion

Human T cells subsets were classified based on the expression of the surface receptor molecules: naïve and T memory cells could be identified by the expression of the CD45RA+ and CD45RO+ isoforms, respectively; Tcm and Tem were discriminated by lymphoid-homing molecules CCR7 and CD62L (L-selectin); Tscm cells had been described as a long-lived memory T cell population which expressed

CD45RO-, CCR7+, CD45RA+, CD62L+, CD27+, CD28+, and IL-7R α +, CD95+. CD95+ indicated that there were some cells with naïve markers that have memory properties and could be used to distinguish Tn from Tscm; CD45RA- was used to identify memory T cell population; CD62L+ stated that cells had limited effector functions, CD62L- represented mediating rapid effector functions [19-24]. So we identified the memory cells by phenotype of major clusters including Tn (CD45RA+CD45RO-CD62L+CD95-), Tscm (CD45RA+CD45RO-CD62L+CD95+), Tcm (CD45RA-CD45RO+CD62L+CD95+), Tem (CD45RA-CD45RO+CD62L-CD95+), Tte (CD45RA+CD45RO-CD62L-CD95+) [18].

In this study, we detected the percentages and absolute counts of T lymphoid subgroups by the single platform technique. There were two reasons: firstly, the precision and difference of detecting the percentages and absolute numbers of lymphocyte subset were different among laboratories. The single-platform was of more accuracy and consistency because of using the known total number of fluorescent microbeads as the standard internal parameters and adding fluorescent labelled antibodies into the trucount tubes, then applied acquisition and analysis software in the flow cytometry to gain accurate data according to the formula as follow.

Table 3. Reference range of absolute counts of T lymphocyte subsets in different gender

Parameters		Male	Female
CD3+ (cells/ μ l)	Median	1179.20	1097.76
	Reference range <i>CI</i> 90%	(743.07–1671.83)	(744.05–1717.67)
CD3+CD4+ (cells/ μ l)	Median	592.17	579.8
	Reference range <i>CI</i> 90%	(369.76–968.89)	(353.70–982.87)
CD4+Tn (cells/ μ l)	Median	24.93	25.46
	Reference range <i>CI</i> 90%	(4.44–70.56)	(6.93–85.49)
CD4+Tscm (cells/ μ l)	Median	107.31	98.84
	Reference range <i>CI</i> 90%	(23.63–326.29)	(12.80–268.27)
CD4+Tcm (cells/ μ l)	Median	156.55	145.2
	Reference range <i>CI</i> 90%	(74.89–299.08)	(79.22–369.68)
CD4+Tem (cells/ μ l)	Median	117.46	114.84
	Reference range <i>CI</i> 90%	(58.48–265.54)	(57.52–319.33)
CD4+Tte (cells/ μ l)	Median	2.46	2.19
	Reference range <i>CI</i> 90%	(0.19–64.98)	(0.20–39.58)
CD3+CD8+ (cells/ μ l)	Median	447.28	378.38
	Reference range <i>CI</i> 90%	(198.21–747.19)	(212.78–645.61)
CD8+Tn (cell/ μ l)	Median	12.59	12.01
	Reference range <i>CI</i> 90%	(1.38–45.99)	(1.14–47.77)
CD8+Tscm (cells/ μ l)	Median	67.60	43.24
	Reference range <i>CI</i> 90%	(6.33–212.74)	(6.10–192.84)
CD8+Tcm (cells/ μ l)	Median	40.10	31.37
	Reference range <i>CI</i> 90%	(13.11–102.13)	(12.63–87.83)
CD8+Tem (cells/ μ l)	Median	91.12	72.71
	Reference range <i>CI</i> 90%	(26.51–248.96)	(21.50–246.29)
CD8+Tte (cells/ μ l)	Median	54.32	43.63
	Reference range <i>CI</i> 90%	(4.41–182.83)	(8.71–216.17)

Table 4. Reference range of percentages of T lymphocyte subsets in different gender

Parameters		Male	Female
CD3+CD4+ % CD3+	Median	52.80%	52.80%
	Reference range <i>CI</i> 90%	(36.94–68.43%)	(37.30–68.40%)
CD4+Tn %CD4+	Median	4.10%	4.50%
	Reference range <i>CI</i> 90%	(0.9–9.10%)	(1.55–12.25%)
CD4+Tscm %CD4+	Median	18.30%	16.90%
	Reference range <i>CI</i> 90%	(3.51–39.29%)	(2.4–38.55%)
CD4+Tcm %CD4+	Median	27.15%	26.10%
	Reference range <i>CI</i> 90%	(14.11–42.23%)	(15.65–46.9%)
CD4+Tem %CD4+	Median	20.35%	20.00%
	Reference range <i>CI</i> 90%	(10.44–40.07%)	(10.55–41.55%)
CD4+Tte %CD4+	Median	0.50%	0.40%
	Reference range <i>CI</i> 90%	(0.01–11.13%)	(0.01–8.1%)
CD3+CD8+ %CD3+	Median	37.80%	35.20%
	Reference range <i>CI</i> 90%	(23.18–53.17%)	(21.95–49.75%)
CD8+Tn %CD8+	Median	3.25%	3.10%
	Reference range <i>CI</i> 90%	(0.4–9.03%)	(0.3–9.65%)
CD8+Tscm %CD8+	Median	15.15%	12.70%
	Reference range <i>CI</i> 90%	(2.21–42.13%)	(1.4–41.85%)
CD8+Tcm %CD8+	Median	9.10%	8.40%
	Reference range <i>CI</i> 90%	(3.4–21.53%)	(2.95–20.9%)
CD8+Tem %CD8+	Median	23.10%	21.30%
	Reference range <i>CI</i> 90%	(6.34–48.8%)	(5.55–49.2%)
CD8+Tte %CD8+	Median	12.40%	10.90%
	Reference range <i>CI</i> 90%	(1.64–38.12%)	(2.55–42.35%)

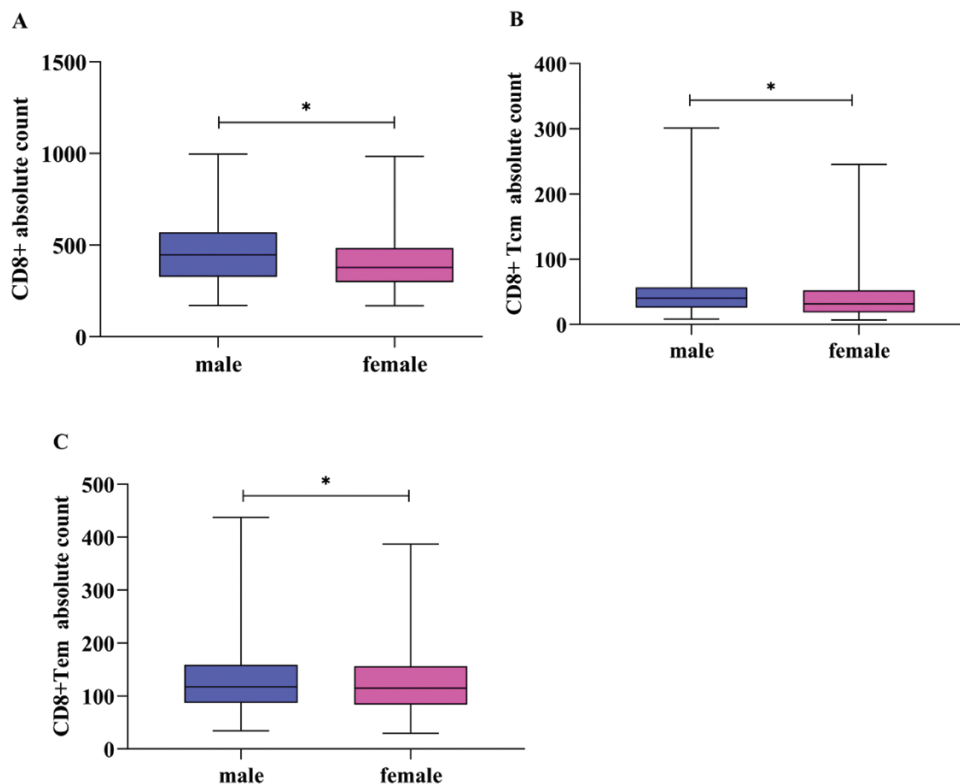


Fig. 4 Comparison of the absolute counts of T lymphocyte subsets in different gender. (A–C) showed that the absolute counts of CD3+CD8+, CD8+Tcm, CD8+Tem in the male group were higher than those in the female group ($P < 0.05$) (* represents significant differences).

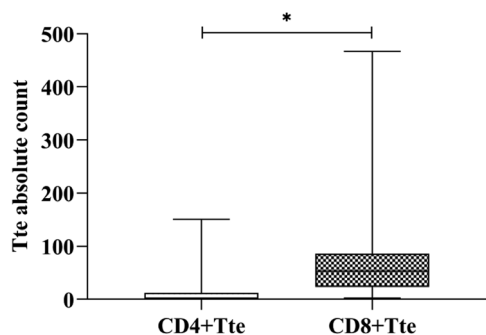


Fig. 5 The absolute count was different in CD3+CD4+Tte and CD3+CD8+Tte. The absolute of Tte in CD8+ were higher than that in CD4+ (* represents significant differences).

$$\text{cells/ul} = \frac{\text{acquired cells} \times \text{total beads}}{\text{acquired beads} \times \text{volume of sample}} \times 100\%$$

Secondly, the clinical significance was different. The percentages of T lymphocyte subsets represented the proportion or composition of each subsets, indicating the development and differentiation of lymphocytes, while absolute counts suggested the proliferation capacity of lymphocytes characterized by the precise amount [12]. The percentage often did not accurately reflect the immune damage to the patients because they did not consider total, which might constantly change in these patients, especially those who were receiving anticancer therapies [12]. In our previous study, we had proved that comparing healthy controls to patients, absolute numbers of CD3+, CD3+CD4+, CD3+CD8+, B and NK cells decreased

in the patients with the non-small cell lung cancer obviously, but the percentages of them were normal [25]. Therefore, it is crucial and urgent to detect both percentages and absolute numbers of lymphocyte subsets in the clinic, it will help us to know the changes in the patient's immunologic function comprehensively, to analyze the clinical condition and predict the curative effect of patients for clinicians [26].

From the result, we could see stem-like cells, such as Tn, Tscm cells decrease with aging. Along with aging, thymus occurs changes that begin during childhood, including a reduction in thymic volume, loss of epithelial cells, increase in perivascular space, and replacement of thymic tissue by fat [27]. Thymus output declines with aging [28], which probably leads to the gradual decline in CD3+CD4+ and CD3+CD8+ naïve T cell numbers, although naïve T cell numbers decline less dramatically than thymocyte numbers [29]. In vitro, researchers had demonstrated that Tscm originated from Tn [24], so it is easier to understand why Tscm decreased with aging, the production of naïve T cells diminished with the involution of the thymus [30–32]. These cells have the ability not only to self-renew but also to differentiate into all subsets of memory and effector T cells [33, 34]. Combined with their longevity, the reserve of Tscm plasticity may play a central role in maintaining immunologic competence with aging [35]. The exhaustion of the Tn and Tscm reservoir suggests that the T-cell pool is a major target of the aging process and may define a parameter possibly related to the life span of humans [29].

In contrast to Tn and Tscm, the absolute counts of CD4+Tcm, CD4+Tem increased with aging, the counts of 45–64 years old and over 65 years old were higher than those in 18–45 years old ($P < 0.05$, Fig. 2C and D), but there was no difference between the group of 45 and 64 years old and over 65 years old

($P > 0.05$). The absolute count of CD8+Tcm in over 65 years old group was more than that in 18–45 years old ($P < 0.05$, Fig. 2G). Tcm and Tem increased with aging and keep less fluctuation over 65 years old [36]. With advancing age, the major goal of T cells shifts to mounting appropriate responses against novel infections and protecting the host against reinfection with common pathogens [37]. When a first stimulus triggers a first response [1], Tn encounters cognate antigen and expand clonally to generate effector cells that migrate to peripheral tissues and eliminate virus and malignant cells [38]. During this effector process, most effector cells become terminally differentiated, termed as short-lived effector cells, while a fraction of effector cells, termed as memory precursor effector cells acquired the ability to survive under the contraction stage of the immune response [22], and further differentiate into a heterogeneous pool of memory cells under optimal developmental conditions, when a second stimulus triggers a second response, more stronger, speedy, durative, and specificity occurs [22].

The majority of effector T cells contract rapidly and are not present in at a steady state, a population of Tte exhibiting CD45RA+CD62L– phenotypes can persist in circulation [39]. Tte cells are mostly present within the CD8+ T cell lineage, exhibiting high capacity for IFN- γ production and low proliferative capacity [40], CD4+Tte cells are rarely detected, some researchers found that the expansion of CD4+Tte cells with cytotoxic function occurs in individuals infected with Dengue virus and was associated with protection [41].

All in all, we should pay more attention to the percentages and absolute counts' changes of T lymphocyte subsets in the clinic simultaneously, for it may give us more important references on treatment.

Conclusion

The reference values of percentages and absolute numbers of naïve T and T memory cell subsets can help doctors to understand the immune state of patients and to evaluate conditions of prognosis then adjust treatment for patients.

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Conflict of interests

The authors declare that they have no potential conflicts of interest.

Author contributions

J.Y. conceptualization, methodology. Y.X., A.L., and W.L. writing – original draft, formal analysis, writing – review and

editing. Y.L., S.Y., G.Z., and Z.Z. data curation, validation. J.S. Y.J., X.L., Y.G., and H.C. resources.

Ethics approval and consent to participate

All clinical and ethical regulations were given the informed consent in accordance with Declaration of Helsinki and the clinical trial was approved by the First teaching hospital of Tianjin University of TCM ethics committee review (TYLL2017[K]002) and registered at the Chinese Clinic Trial Registry (ChiCTR-IOR-17014139).

Availability of data and materials

All data generated and analyzed during this study are available from the corresponding author in response to reasonable requests.

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