

Article

Reference ranges and age-related changes of peripheral blood lymphocyte subsets in Chinese healthy adults

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This study was performed to build region-specific reference ranges of peripheral blood lymphocyte subsets for Chinese healthy adults from the young to the elderly and analyze the trends of changes in lymphocyte subsets for evaluating the impact of age on the values. 151 healthy adults aged 19–86 were recruited based on the SENIEUR protocol. Three sets of reference ranges were finally built applicable for the healthy young (19–44 years), middle-aged (45–64 years) and elder adults (≥65). Comparisons in parameters among the three cohorts showed that a statistically significant increase in CD16CD56⁺ NK cell was observed between the middle-aged and elder cohorts, whereas for the majority of the parameters, a significant decline was observed between the young and the middle-aged cohorts. Further results showed that inverse correlations were observed between the age and CD19⁺ B, CD3⁺ T, CD3⁺CD4⁺ T, CD4⁺CD45RA⁺CD62L⁺ naïve T cell and CD4⁺CD28⁺/CD4⁺, while the positive one was identified between the age and the NK cell. These significant changes of the most of immune parameters provided evidence for immunosenescence. Notably, T cell activation markers of CD8⁺CD38⁺ and CD8⁺HLA-DR⁺ showed reverse trends of association with age, which provides a clue for further researches on the mechanisms underlying the paradoxical clinical presentation of the elder patients.

lymphocyte subsets, age, reference range, immunosenescence

With tremendous advances in the field of flow cytometry, the immunophenotyping of peripheral blood lymphocytes has evolved into the most important tool in the evaluation of immune status in patients with congenital and acquired immune deficiency syndromes^[1], as well as other immune-mediated diseases. Establishment of reference ranges for peripheral blood lymphocyte subsets in healthy populations is the fundamental element for the clinical applications of immunophenotyping in monitoring immunological changes. At the beginning, it was widely conducted for the enumerating of CD4⁺ T cell counts in the management of HIV infection, so most of the results were restricted to young adults. However, human aging is associated with progressive decline in immune function, which often leads to higher morbidity

and mortality due to disease and contributes to increased susceptibility to infection, autoimmune disease and cancer^[2]. Many previous studies revealed variations in the normal ranges for lymphocyte subsets according to age^[3–5].

A few studies have been conducted to observe age-

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related changes in lymphocyte subsets and reference ranges were built for the middle-aged or the elderly in different countries. However, the age ranges of the subjects in most studies were relatively narrow, especially for the elderly. Accumulating results also suggest that the genetic and environmental differences among different ethnical populations lead to variable findings, which was also observed among the populations of neighborhood countries in Asia^[6] Region-specific reference values for lymphocyte subpopulations in different age groups, especially in elder adults, are lacking in China. A systemic examination of age-related changes is necessary for discovering the underlying mechanisms of immunosenescence and the assessment of immune-related disease in different age groups.

Therefore, in this study, we managed to establish reference ranges for lymphocyte subsets in healthy adults with a wide range of age. The differences among three age groups from young adults to the elderly were compared and the trends of changes in lymphocyte subsets were observed for evaluating the impact of age on the values.

1 Materials and methods

1.1 Subjects

The subjects were adults above age 18 and were recruited between February and September 2007. They were all healthy volunteers strictly based on defined criteria from the SENIEUR protocol guideline^[7]. Furthermore, they were also screened for serum levels of tumor markers, including CEA, CA50, CA199, CA242, PSA(for male), CA125(for female), to rule out potential patients with cancers, such as prostate cancer, which develop very slowly and might be overlooked despite a long-term clinical observation.

Prior to inclusion into the study, all participants took periodical check-up for health for at least 3 years in Peking Union Medical College Hospital and individuals eligible were then followed up 6 months later after the time of phlebotomy for a similar screening process. According to the definition made by the Ministry of Health of the People's Republic of China, a human population 65 years of age or above is considered elderly, groups between 18 and 45 years of age are considered young and the ones from 45 to 64 are considered the middle-aged. Informed consents were obtained from all

subjects, and the Ethical Committee of Peking Union Medical College Hospital approved the protocol.

1.2 Lymphocyte immunophenotyping

Immunophenotyping was analyzed by use of three-color flow cytometry (Epics XL flow cytometry; Bechman Coulter, USA) as previously described^[8,9]. Freshly collected EDTA-anticoagulated whole blood was incubated with a panel of monoclonal antibodies (MAb) directed against fluorescein isothiocyanate/phycoerythrin/peridinin chlorophyll protein combinations of CD3/CD8/CD4, CD3/CD16 plus CD56/CD19, HLA-DR/CD38/CD8, CD28/CD8/CD4, CD62L/CD45RA/CD4 and isotype controls (Immunotech, France). Absolute counts of lymphocyte subset cells were then calculated using a dual-platform method with the white blood cell counts and lymphocyte differentials obtained from the results of blood routine tests.

1.3 Statistical analysis

Statistical analysis was performed by use of SPSS software (SPSS[®] for Windows[™] version 11.5, SPSS Inc., Chicago, IL, USA). Kolmogorov-Smirnov was used for the distribution test. Comparisons among variables were performed using one-way analysis of variance, and the least significant difference procedure test was used for further multiple comparisons in analysis of variance. Association between variables and age was tested using a non-parametric Spearman's rank correlation test. A difference with P value of less than 0.05 was considered to be significant.

2 Results

A total of 151 healthy adults (100 males, 51 females, mean age \pm SD: 52 \pm 18 years old) who fulfilled the study criteria were included in this study. They ranged in age from 19 to 86 years old. Of these, 52 (34.4%) were elder individuals (65 years old and above, 40 males, 12 females, mean age \pm SD: 72 \pm 5 years old), 47 (31.1%) were middle-aged ones (45—64 years old, 29 males, 18 females, 54 \pm 6 years), and 52 (34.4%) belonged to young adults (19—44 years old, 31 males, 21 females, mean 31 \pm 6 years). The Chi-square test demonstrated that the gender was balanced among the three cohorts ($P=0.128$) and age distributions by every decade are listed in Table 1.

Medians and 95% reference intervals (percentile 2.5

Table 1 Age distribution by every decade

	Younger than 25	25—34	35—44	45—54	55—64	65—74	75 and above
<i>n</i>	8	26	18	25	22	35	17
% in whole	5.3	17.2	11.9	16.6	14.6	23.2	11.3

Table 2 Medians and 95% reference intervals for peripheral blood lymphocyte subsets in different age groups^{a)}

		All (<i>n</i> =151)	Young (<i>n</i> =52)	Middle-aged (<i>n</i> =47)	Elder (<i>n</i> =52)	<i>P</i> value
Lymphocyte counts	median	1926	2065	1882	1854	0.062
	interval	1050—90	1409—3425	1016—3730	975—3498	
CD19 ⁺ B percentage	median	10.9	12.6	10.0	9.8	0.030
	interval	4.4—21.2	6.4—30.6	4.2—19.7	2.4—24.3	
CD19 ⁺ B counts	median	209	254	190	182	0.004
	interval	74—534	104—571	77—566	39—544	
CD16CD56 ⁺ NK percentage	median	15.1	13.4	13.9	21.2	0.000
	interval	3.7—46.1	4.2—35.6	3.5—40.4	4.1—49.8	
CD16CD56 ⁺ NK counts	median	297	269	283	390	0.003
	interval	63—1013	96—872	53—985	71—1340	
CD3 ⁺ T percentage	median	69.4	72.1	70.4	63.6	0.000
	interval	43.7—80.5	49.4—80.8	52.4—80.4	38.7—81.9	
CD3 ⁺ T counts	median	1300	1417	1316	1086	0.001
	interval	711—2353	826—2409	639—2780	615—2493	
CD3 ⁺ CD4 ⁺ T percentage	median	37.8	38.8	38.1	34.6	0.215
	interval	22.5—55.1	25.1—64.0	19.8—54.0	19.6—55.2	
CD3 ⁺ CD4 ⁺ T counts	median	734	769	762	653	0.106
	interval	368—1632	442—1645	259—1946	291—1730	
CD3 ⁺ CD8 ⁺ T percentage	median	24.8	26.6	26.5	21.7	0.012
	interval	11.2—43.1	16.7—2	15.9—42.3	8.2—44.4	
CD3 ⁺ CD8 ⁺ T counts	median	507	532	517	385	0.001
	interval	201—931	321—1166	208—825	173—1114	
CD4 ⁺ CD45RA ⁻ /CD4 ⁺	median	64.5	60.5	65.2	68.7	0.007
	interval	36.0—93.4	36.4—77.9	28.5—94.0	36.0—95.3	
CD4 ⁺ CD45RA ⁻	median	439	455	436	419	0.837
	interval	230—1104	248—1114	177—862	191—1188	
CD4 ⁺ CD45RA ⁺ CD62L ⁺ /CD4 ⁺	median	31.3	33.8	29.2	25.4	0.009
	interval	5.7—63.5	17.4—61.5	5.2—68.5	2.2—62.7	
CD4 ⁺ CD45RA ⁺ CD62L ⁺	median	232	296	192	173	0.009
	interval	33—601	117—586	40—716	25—688	
CD4 ⁺ CD28 ⁺ /CD4 ⁺	median	93.6	96.2	92.5	89.7	0.000
	interval	65.3—99.0	81.5—99.5	56.9—98.7	40.4—98.6	
CD8 ⁺ CD28 ⁺ /CD8 ⁺	median	50.7	62.9	51.0	42.3	0.000
	interval	17.7—86.5	34.9—87.4	21.5—86.7	13.7—84.3	
CD8 ⁺ HLA-DR ⁺ /CD8 ⁺	median	20.9	7.6	24.4	30.3	0.000
	interval	1.5—48.3	0.6—32.4	1.6—42.0	6.2—51.8	
CD8 ⁺ CD38 ⁺ /CD8 ⁺	median	19.4	41.6	15.7	13.3	0.000
	interval	4.2—62.3	10.3—67.0	2.4—66.7	5.2—49.7	
CD4 ⁺ /CD8 ⁺	median	1.56	1.44	1.56	1.81	0.070
	interval	0.63—3.49	0.57—3.28	0.55—3.30	0.62—5.97	

a) Absolute counts are given in cells per microliter. Intervals are given as 2.5 and 97.5 percentiles. Young, healthy young adults, 19—44 years old; Middle-aged, healthy middle-aged individuals, 45—64 years old; Elder, healthy elder individuals, 65 years old and above; CD, cluster of differentiation; HLA, human leukocyte antigen; DR, D-related.

—97.5) were calculated for the complete sets of lymphocyte subsets. The results are shown in Table 2. On the whole, there were significant differences among the three cohorts for most parameters. Age did not seem to influence CD4⁺ T cell counts CD4⁺CD45RA⁻ memory T cell counts, CD3⁺CD4⁺/CD3⁺ and CD4⁺/CD8⁺ significantly in our study, although a trend of decrease in CD4⁺ T cell count ($r=-0.239$ $P=0.003$) and increase in CD4⁺/CD8⁺ ($r=0.160$ $P=0.050$) was observed with age. The further multiple comparisons in parameters with significant difference among the three cohorts showed that different patterns existed. For the absolute count and the percentage of CD16CD56⁺ NK cell, significant increases with age happened between the middle-aged and the elder cohorts ($P=0.002$ and 0.000 , respectively), but no significant difference existed between the young and the middle-aged ($P=0.744$ and 0.725 , respectively). However, for the majority of the parameters, such as the absolute counts of CD19⁺ B, CD3⁺T and CD3⁺CD8⁺ T cells, and the percentages of CD19⁺ B, CD4⁺CD45RA⁺CD62L⁺/CD4⁺, CD4⁺CD28⁺/CD4⁺ and CD8⁺CD38⁺/CD8⁺, significant differences were observed between the young and the middle-aged cohorts ($P=0.005$, 0.043 , 0.040 , 0.022 , 0.048 , 0.007 and 0.000 , respectively), but not between the middle-aged and the elderly ($P=0.999$, 0.076 , 0.121 , 0.952 , 0.333 , 0.082 and 0.063 , respectively). For the percentages of CD8⁺CD28⁺/CD8⁺ and CD8⁺HLA-DR⁺/CD8⁺, significant differences existed between each two cohorts.

We then observed the associations between a set of parameters and age (results shown in Figure 1). Inverse correlations were observed between the age and CD19⁺ B, CD3⁺ T, CD3⁺CD4⁺ T, CD4⁺CD45RA⁺CD62L⁺ naïve T cell and CD4⁺CD28⁺/CD4⁺, while the positive one was identified between the age and CD16CD56⁺ NK cell. More interestingly, the T cell activation markers of CD8⁺CD38⁺ and CD8⁺HLA-DR⁺ showed reverse trends of association with age.

For comparisons with the results of previous studies, in which the subjects were divided into different groups by decade, we further observed the age-related changes by decade for lymphocyte subsets (results shown in Figure 2). Although similar trends were obtained for all parameters, fluctuant results could be observed in different parts of the curves.

3 Discussion

In this study, we managed to obtain reference ranges for

lymphocyte subsets in healthy adults with different age ranges. These reference ranges are applicable to adults between the ages of 19 and 85 years, which is the majority of clinically relevant adult population. As expected, the results obtained from Chinese adults are different from the results of populations studied in other countries, even when the comparisons were made between the populations with similar age ranges. It seemed that Chinese population has lower CD4⁺T cell counts than Caucasian race (Chinese vs Caucasian: age range 19—86 vs 19—85 years old; median 734 vs 870 cells/ μ L; interval 368—1632 vs 490—1640 cells/ μ L)^[10], but has higher CD4⁺ T cell counts than African population (Chinese vs African: age range 19—44 vs 15—45 years old; interval 442—1645 vs 370—1240 cells/ μ L)^[11]. Results from other Asian countries appear to demonstrate higher absolute counts of B, NK, T and CD8⁺ T cells but a similar level of CD4⁺ T cell counts, even when compared with the Chinese subset in Singaporean population (Chinese vs Chinese in Singapore: age range 19—65 vs 16—65 years old; CD3+T cell mean \pm SD 1428 \pm 424 vs 1547 \pm 453; CD3+CD4+T 797 \pm 307 vs 812 \pm 255; CD3+CD8+T 543 \pm 183 vs 629 \pm 235; CD19+B 242 \pm 111 vs 330 \pm 132; CD16CD56+ NK 313 \pm 182 vs 425 \pm 244)^[6]. The genetic and environmental variations between populations might be possible causes of the differences in lymphocyte subsets between populations, and variable CD4⁺ T cell results may be attributable to different nutrition status. However, our results suggested that region-specific reference ranges for lymphocyte subsets were necessary in China. Further solid comparison results need well-designed trials using the methodology with good inter-laboratory comparability.

Controversial findings have been reported about the impact of age on peripheral blood immunological markers. Some showed that the CD4⁺ T cell increased with age^[12,13], while others showed that the CD4⁺ T cell decreased with age^[14–16], or no significant variation according to age at all except CD8⁺ T cell decreased with age^[6]. However, a thorough review of these studies revealed that the inconsistency arose because cohorts in these studies were within different narrow age ranges, some of which were between 20 and 50 years old for the purpose of management of HIV infection and some were even narrower^[11,17–19]. As shown in Figure 2, the dynamic changes of these parameters by every decade

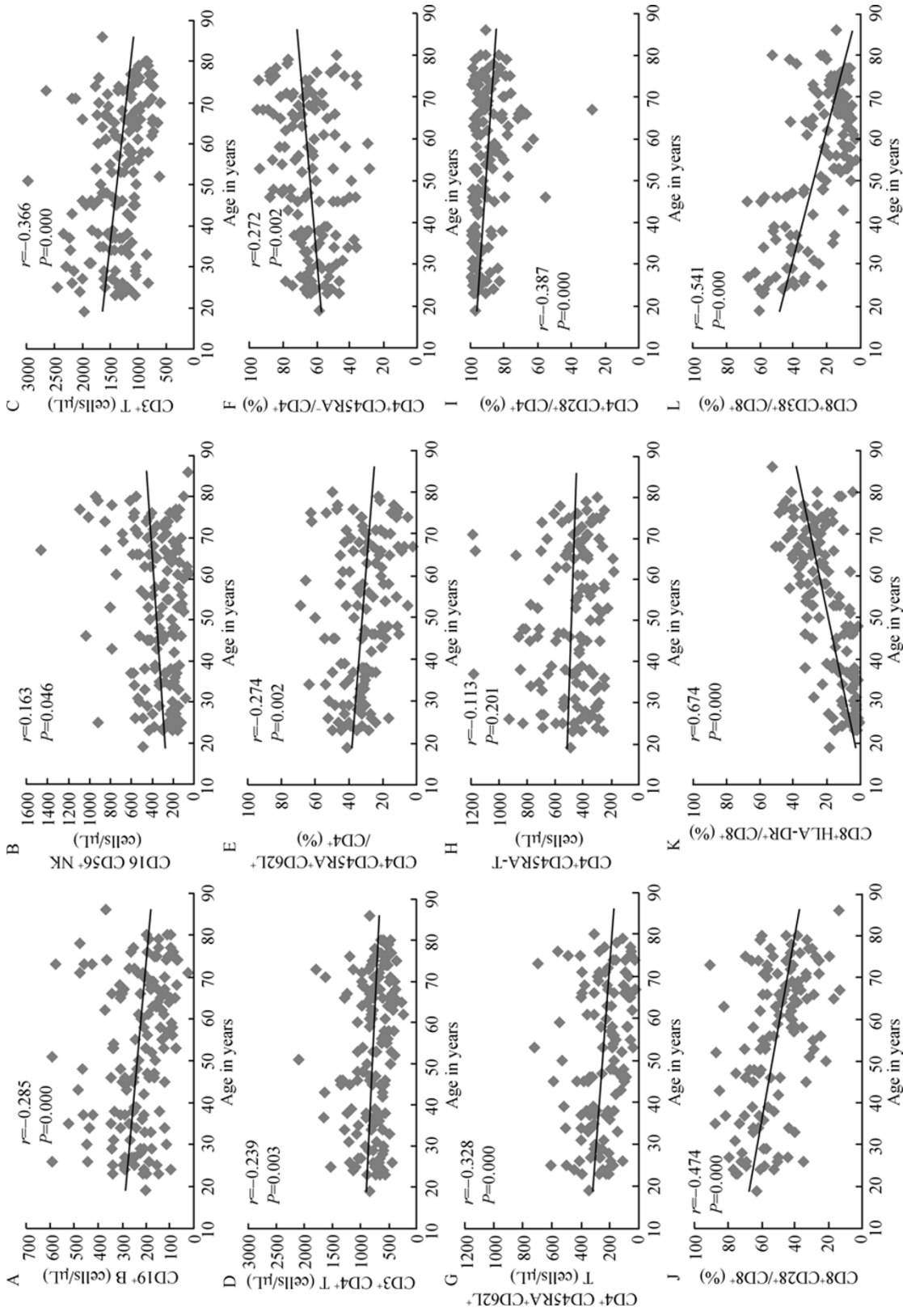


Figure 1 Scatter diagrams showed correlations between age and absolute counts or percentages of lymphocyte subsets in all healthy adults. A, CD19⁺ B cell count; C, CD3⁺ T cell count; D, CD3⁺CD4⁺ T cell count; E, percentage of CD4⁺CD45RA⁺CD62L⁺/CD4⁺ naive T cell; G, CD4⁺CD45RA⁺CD62L⁺ naive T cell count; H, CD4⁺CD45RA⁻ memory T cell; I, percentage of CD4⁺CD28⁺/CD4⁺; J, percentage of CD8⁺CD28⁺/CD8⁺ and L, percentage of CD8⁺CD38⁺/CD8⁺ were inversely associated with age. And significant positive correlations were found only between the age and B, CD16CD56⁺ NK cell count; F, percentage of CD4⁺CD45RA⁻/CD4⁺ memory T cell; and K, percentage of CD8⁺HLA-DR⁺/CD8⁺. P values shown in the figure were determined by the Spearman rank correlation test. The solid line represents a regression line.

were fluctuant, so it would be easy to draw variable conclusions when observing different parts of the curves.

In our investigation, inverse correlations were identified between the age and the parameters of B, T, CD4⁺ T, naïve T cell and CD4⁺CD28⁺/CD4⁺, which provided support for the immunosenescence. Furthermore, our results revealed different patterns of age-related dynamics in these parameters. A significant decline in parameters like the CD19⁺ B, CD3⁺T and CD3⁺CD8⁺ T cell counts, and the percentages of CD19⁺ B, CD4⁺CD45RA⁺ CD62L⁺/CD4⁺, CD4⁺CD28⁺/CD4⁺ and CD8⁺CD38⁺/CD8⁺ was observed only in the age range from the young to the middle-aged. Moreover, a signifi-

cant increase in NK cell counts and ratios happened in the age range from the middle-aged to the elderly. These results may reflect different mechanisms underlying the immunosenescence, and the characteristics of immune status in different age ranges may play an important role in the incidence of specific immune-mediated diseases.

A significant increase in the T-cell activation marker of CD8⁺HLA-DR/CD8⁺ and a converse trend in another activation marker of CD8⁺CD38⁺/CD8⁺ were noted in our analysis. It has been addressed by few previous studies. Deterioration of the immune system with aging is believed to contribute to increased morbidity and mortality in elder patients. Previous studies sug-

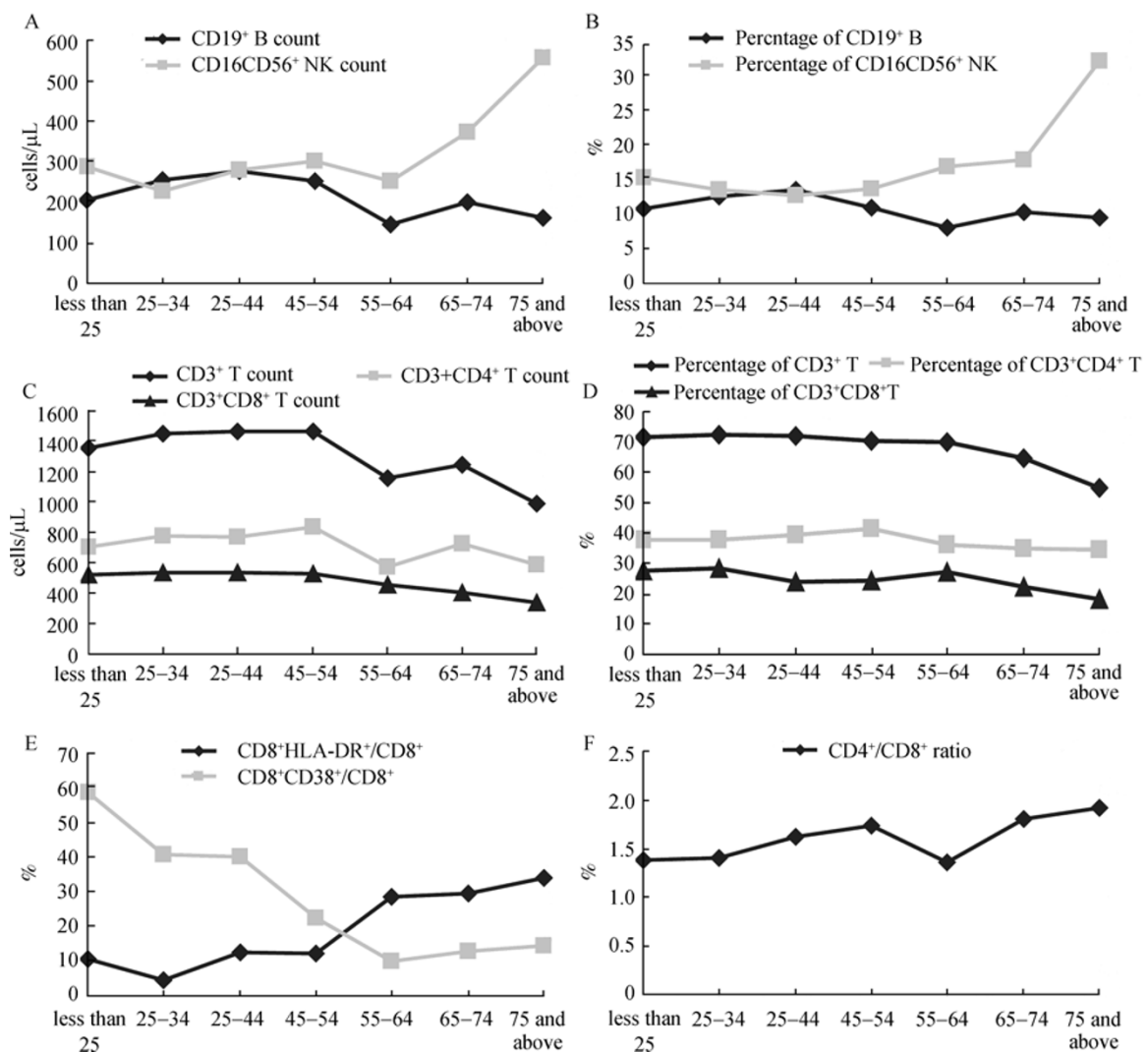


Figure 2 Linear diagrams showed age-related changes by decade for lymphocyte subsets of A, CD19⁺ B and CD16CD56⁺ NK cell counts; B, percentages of CD19⁺ B and CD16CD56⁺ NK cell; C, CD3⁺T, CD3+CD4⁺ T and CD3⁺CD8⁺ T cell counts; D, percentages of CD3⁺T, CD3+CD4⁺ T and CD3⁺CD8⁺ T cell; E, percentages of CD8⁺HLA-DR⁺/CD8⁺ and CD8⁺CD38⁺/CD8⁺; F, percentages of CD4⁺/CD8⁺.

gested that the major predictor of mortality in the elderly is lung function^[20]. Evidence for immune dysregulation in the aging human lung showed the presence of low-grade inflammation in many apparently clinically normal lungs in the elderly compared to the young^[21]. Paradoxically, immunosenescence may sometimes contribute to decreased pathology in elderly individuals, as in the lesser acute rejection seen in kidney or liver transplantation^[22,23] and in the decreased allergic reactions^[24] or incidence of systemic lupus erythematosus^[25]. Activation-induced apoptosis was naturally thought to play an important role in the process and similar phenomena may be reflected in pathological states such as chronic phase of HIV infection^[26]. Additionally, our results on the dynamics of different T-cell activation markers with age provided clues for the possible mechanism underlying the paradoxical clinical presentation among the elderly. However, more investigations are necessary to define the precise clinical relevance of T-cell immunosenescence.

Previous studies also demonstrated the gender-related differences in immune cell numbers or percentages^[10,12,14,17,27,28]. The underlying mechanisms may be secondary to the differential influences of sex hormones^[29], which may include that androgens accelerate thymocyte apoptosis and therefore shape the peripheral T cell repertoire^[30,31]. However, the impact of gender differences on lymphocyte subsets was not addressed in our study, despite the fact that all three cohorts were gender balanced. It was concerned that the relatively small number of females in each cohort might lead to less reliable results. A larger number of volunteers should be collected for further research.

There are several possible limitations in our study. Firstly, the recruitment of the subjects was mainly based on the SENIEUR protocol, which has strict exclusion

criteria, limiting studies to only the healthiest individuals. As a consequence, it may rule out 85% of the aged population^[32,33]. Although that could guarantee ruling out individuals with altered immune responses as a result of underlying infection or pathology, it is also possible that the altered immune status was age-related, which predisposed the elder ones to developing infection or pathology. So the recruitment of healthy adults with a wide range of age using the SENIEUR protocol may lead to some bias in reference values especially for the elder cohort and hamper their clinical application. Secondly, absolute lymphocyte values were calculated using a dual-platform instead of a single-platform method. It is still unclear whether the reference values could be applicable for the results by the other method, although some previous studies have shown no differences in results between the single- and the dual-platform methods^[17,34]. Thirdly, the majority of the subjects was from the Han population. Considering probable ethnic variation, it should be cautious in the application of the results in minority populations in China.

In summary, our investigation leads to the establishment of reference ranges for peripheral blood lymphocyte subsets in Chinese healthy adults. The three region-specific values for different age groups can be used to guide patient management and interpretation of clinical research findings in not only HIV infection but also for a variety of immune-modulating disease entities. The converse trends of T cell activation markers with age may play a certain role in the paradoxically clinical presentation of immunosenescence.

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