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Reference Ranges for Lymphocyte Subsets among Healthy Hong Kong Chinese Adults by Single-Platform Flow Cytometry

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Race, age, sex, and environmental conditions have significant impacts on lymphocyte subset values. It is important to establish the local reference ranges from healthy and non-HIV-positive adults in the local population for clinical decision making. In this study, the reference ranges for lymphocyte subsets among Chinese adults were established by analysis by single-platform flow cytometry of the lymphocyte compositions of 273 healthy adult blood donors between 17 and 59 years of age. The 95% reference ranges for $CD3^+$ T cells, $CD3^+$ $CD4^+$ T helper cells, and $CD3^+$ $CD8^+$ T suppressor cells are 723 to 2,271 cells/μl, 396 to 1,309 cells/μl, and 224 to 1,014 cells/μl, respectively. The 95% reference ranges for $CD19^+$ B cells and $CD56^+$ NK cells are 118 to 645 cells/μl and 61 to 607 cells/μl, respectively. Significant gender and age differences in the lymphocyte subsets have been demonstrated. Our results have also shown that the T-lymphocyte compositions in Hong Kong Chinese were comparable to those of other Asian populations but were different from those of Caucasians.

Lymphocyte subset enumeration is useful in the evaluation of hereditary and acquired immunodeficiency, assessment of immune reconstitution post-allogeneic stem cell transplantation, and also for monitoring CD4 count in HIV and AIDS patients. In particular, the CD4 count serves as a guide for initiation of antiretroviral therapy and prophylactic treatment of opportunistic infections (1–3) because it can provide valuable information for treatment response and disease progression. As a result, immunophenotyping of lymphocyte subsets by flow cytometry has been an indispensable tool in the management of HIV and AIDS patients. Various studies have shown that the reference ranges of lymphocyte subsets are subject to influences by age, sex, ethnicity, and environment (4–17). Flow cytometry is the "gold standard" for the enumeration of lymphocyte subsets, and it is important to establish the local reference ranges for interpretation of laboratory results and clinical decision making (13). Most of the published reference ranges were established by dual-platform flow cytometry (3, 5, 6, 9–12, 14–17).

Healthy blood and apheresis donors from the local blood transfusion service were recruited for the establishment of the reference ranges of lymphocyte subsets by the use of single-platform flow cytometry. Single-platform flow cytometry allows simultaneous identification and enumeration of total $CD3^+$ T cells, dual $CD3^+$ $CD4^+$ T helper cells, dual $CD3^+$ $CD8^+$ T suppressor cells, $CD19^+$ B cells, and $CD56^+$ natural killer cell population percentages and absolute cell counts. Further manipulation of the specimens to obtain absolute white cell count and differential count by a separated automatic hematology cell counter are avoided by the incorporation of a known quantity of microfluorospheres in the flow cytometry step (2). The whole procedure is more robust, convenient, and reproducible than the traditional dual-platform flow cytometry. Gender- and age-related differences in the lymphocyte subsets were evaluated. The results were also compared with those from different ethnic groups. The influence of sex and age on the lymphocyte composition and comparison with data from both Asian and non-Asian populations will be discussed.

MATERIALS AND METHODS

Study population. Healthy blood or apheresis donors from the Hong Kong Red Cross and Blood Transfusion Service were recruited for the establishment of reference ranges of lymphocyte subsets in the Queen Elizabeth Hospital, Hong Kong, during the period from October 2009 to January 2010. Informed consent was obtained. A questionnaire was completed by each donor to exclude those who have high-risk behaviors for HIV or AIDS, recent infections, significant medical illnesses, or long-term medications, including Chinese herbal medicine. All specimens were screened negative for hepatitis B virus (HBV), hepatitis C virus (HCV), HIV-1, and HIV-2 by serological studies and by nucleic acid tests (NAT). Blood samples were collected into EDTA-anticoagulated Vacutainers and transported to the hematology laboratory within 2 h of collection for flow cytometry analysis. All specimens were processed within 6 h of venipuncture.

Reagents, cell preparation, staining, and analysis. A single platform with a lyse-no-wash procedure for flow cytometric analysis of lymphocyte subsets by the FC500 flow cytometer (Beckman Coulter) (1) was performed with the use of two sets of four-color monoclonal antibody combinations (Cyto-Stat tetraCHROME; Beckman Coulter): (i) CD45 (fluorescein isothiocyanate [FITC/CD4 (phycoerythrin [RD1])/CD8 (phycoerythrin-Texas Red-x [ECD])/CD3 (phycoerythrin-cyanine 5 [PC5]) and CD45 (FITC)/CD56 (RD1)/CD19 (ECD)/CD3 (PC5). They were labeled as tubes 1 and 2, respectively. One hundred microliters of donors' samples was incubated with 10 μl of the monoclonal antibodies. Red cells were lysed with the ImmunoPrep reagent system and TQ-Prep workstation (Beckman Coulter).

Briefly, 100 μl of Flow-count fluorospheres (Beckman Coulter) was added to each staining tube. The preparation was analyzed on FC500 flow cytometer system (Beckman Coulter) with the CXP software (version 2.2) and tetraCXP SYSTEM software (version 2.1) packages. The reagents and

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TABLE 1 Reference ranges of lymphocyte subsets obtained from 273 healthy blood and apheresis donors

Parameter[a]	Result from: Present study				
	Total (n = 273)	Male (n = 150)	Female (n = 123)	P value for male vs female[b]	1996 study by Kam et al. (12)
Age (yr)					
Mean	36.5	40	33		32
Median	37	42	29		
SD	11.3	10	11		
No. of $CD3^+$ cells/μl (%)					
Mean	1,362 (71.15)	1,316 (70)	1,418 (72.52)		
SD	382 (6.78)	354 (7.33)	407 (5.78)		
Reference range	723–2,271 (56.09–84.32)	721–2,102 (53.01–84.04)	710–2,363 (61.51–84.39)	0.027* (0.002*)	672–2,368 (54.8–83)
No. of $CD3^+$ $CD4^+$ cells/μl (%)					
Mean	760 (40.25)	735 (39.69)	790 (40.93)		
SD	233 (6.36)	220 (6.62)	245 (5.99)		
Reference range	396–1,309 (28.06–53.39)	393–1,279 (26.58–53.13)	397–1,320 (29.71–53.84)	0.52 (0.108)	292–1,366 (23.1–51)
No. of $CD3^+$ $CD8^+$ cells/μl (%)					
Mean	515 (27.24)	496 (26.71)	539 (28.88)		
SD	192 (6.62)	196 (7.46)	185 (5.37)		
Reference range	224–1,014 (16.37–42.65)	219–1,015 (15.14–45.99)	239–1,039 (17.52–40.81)	0.68 (0.146)	240–1,028 (17.9–47.5)
No. of $CD19^+$ cells/μl (%)					
Mean	298 (15.35)	296 (15.42)	300 (15.27)		
SD	124 (4.54)	130 (4.86)	116 (4.12)		
Reference range	118–645 (7.19–25.85)	92–671 (6.83–25.76)	121–634 (8.27–26.07)	0.791 (0.794)	82–560 (5.1–20.8)
No. of $CD56^+$ cells/μl (%)					
Mean	229 (11.78)	247 (12.81)	207 (10.52)		
SD	132 (5.69)	149 (6.42)	106 (4.35)		
Reference range	61–607 (3.66–26.74)	82–648 (4.76–32.08)	53–525 (3.31–21.94)	0.013* (0.001*)	130–938 (7.1–38)
CD4/CD8 cell ratio					
Mean	1.59	1.64	1.55		
SD	0.55	0.61	0.47		
Reference range	0.71–2.82	0.70–2.97	0.78–2.92	0.21	0.5–1.8

[a] Reference ranges are defined as 95% of the population.
[b] P values for comparison of the male and female populations are shown. *, statistically significant result.

the data acquisition software were approved by the FDA/CE for *in vitro* diagnostic (IVD) use in $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ lymphocyte enumeration. Six thousand and 10,000 events were acquired for tubes 1 and 2, respectively. Lymphocytes were identified by their strong CD45 expression and low side scatter. T helper cells, T suppressor cells, B cells, and NK cells were identified by expression of $CD3^+$ $CD4^+$, $CD3^+$ $CD8^+$, $CD19^+$, and $CD56^+$, respectively.

Quality assurance. The optical alignment and fluidic stability were calibrated daily using Flow-Check fluorospheres (Beckman Coulter), and the monoclonal antibody reactivity was monitored daily by Immuno-Trol (Beckman Coulter) control cells. Compensation of spectral overlap was established by staining Cyto-Comp cells with the Quick-COMP 4 reagent kit. Signal from each specific fluorochrome in the respective fluorescence detector was analyzed on a single-parameter histogram. Our laboratory meets the international standards of the external quality assurance program on lymphocyte subset enumeration organized by the United Kingdom National External Quality Assessment Scheme (UK NEQAS).

Statistical analysis. The means, medians, standard deviations (SD), and reference ranges of the percentages and absolute counts of $CD3^+$ T cells, $CD3^+$ $CD4^+$ T helper cells, $CD3^+$ $CD8^+$ T suppressor cells, $CD19^+$ B cells, and $CD56^+$ NK cells were calculated using the Statistical Package for Social Sciences (SPSS) software version 18. The reference ranges were defined as 95% of the population. The impact of sex and age on lymphocyte subsets was evaluated by Mann-Whitney test or Student's t tests (without assuming equal variance). Results were considered statistically significant at a P value of <0.05.

RESULTS

A total of 273 healthy donors were enrolled during the period from October 2009 to January 2010. Among them, 150 (55%) were male and 123 (45%) were female. Their ages ranged from 17 to 59. A total of 155 (male/female ratio of 0.8) of the donors were less than 40 years old, while 118 (male/female ratio of 2.2) were ≥40 years of age (see below). All were ethnic Chinese. The means, standard deviations, and reference ranges for the percentages of $CD3^+$ T cells, $CD3^+$ $CD4^+$ T helper cells, $CD3^+$ $CD8^+$ T suppressor cells, $CD19^+$ B cells, and $CD56^+$ NK cells, the absolute counts of $CD3^+$ T cells, $CD3^+$ $CD4^+$ T helper cells, $CD3^+$ $CD8^+$ T suppressor cells, $CD19^+$ B cells, and $CD56^+$ NK cells, and the CD4/CD8 cell ratios are shown in Table 1. The 95% reference ranges for

TABLE 2 Comparison of lymphocyte subsets' reference ranges in different ethnic groups

Lymphocyte population	Reference range for population				
	Chinese[a]				
	Hong Kong (n = 273)	Beijing (n = 151) (11)	Asian (Singapore)[b] (n = 232) (8)	European (Germany)[a] (n = 100) (9)	African (n = 2,570) (18)
CD3					
%	56.09–84.32	43.7–80.5	49–80	53–83	
Count/μl	723–2271	711–2,353	796–2,679	780–2,240	
CD4					
%	28.06–53.39	22.5–55.1	23–48.2	30–59	
Count/μl	396–1,309	368–1,632	401–1,451	490–1,640	365–1,571
CD8					
%	16.37–42.65	11.2–43.1	13.4–41	10–40	
Count/μl	224–1,014	201–931	243–1,206	170–880	145–884
CD4/8 ratio	0.71–2.82	0.63–3.49	0.69–2.83	0.9–5.0	0.7–5.3
CD19					
%	7.19–25.85	4.4–21.2	7.0–28.0	5–21	
Count/μl	118–645	74–534	133–714	80–490	
CD56					
%	3.66–26.74	3.7–46.1	6.0–37.0	5–32	
Count/μl	61–607	63–1,013	115–1,009	80–690	

[a] Reference ranges are defined as 95% of the population in these studies.
[b] Reference ranges are defined as 95% of the population, with the exception of CD4 and CD8 percentages, which are defined as means ± 2SD.

the absolute counts of $CD3^+$ T cells, $CD3^+$ $CD4^+$ T helper cells, and $CD3^+$ $CD8^+$ T suppressor cells are 723 to 2,271 cells/μl, 396 to 1,309 cells/μl, and 224 to 1,014 cells/μl, respectively. Their distributions have Kurtosis values of 0.095, 0.106, and 2.293, respectively. The 95% reference ranges for the absolute counts of $CD19^+$ B cells and $CD56^+$ NK cells are 118 to 645 cells/μl and 61 to 607 cells/μl, respectively. Both distributions have Kurtosis values of 2.423 and 4.66, respectively. Gender difference was demonstrated in the percentages and absolute counts of $CD3^+$ T cells and $CD56^+$ NK cells ($P < 0.05$ in both cases). Such differences were not observed for the $CD4^+$ T helper cells and $CD8^+$ T suppressor cells. The absolute count for $CD56^+$ NK cells was lower than that reported previously in the Hong Kong Chinese population (12), which was established 15 years ago (Table 1). Comparison of our data was performed among the published data from different ethnic groups (Table 2). Our values were comparable to those of the Chinese population established in other parts of China (10, 11). They were also similar to those in other Asian populations (8, 17). The reference ranges, however, were different from the reported Caucasian ranges (9) and were also different from the African data, although data from the latter ethnic group are limited (13).

The reference ranges for the lymphocyte subsets were compared between members of the population less than 40 years of age and those 40 years of age or older to evaluate the impact of age on various lymphocyte subpopulations (Table 3). The percentages of $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD56^+$ NK cells, the absolute counts of $CD3^+$ T cells and $CD8^+$ T cells, and the CD4/CD8 cell ratios were found to be different between the two age groups ($P < 0.05$ in all cases).

DISCUSSION

In this study, we have established the updated reference ranges for lymphocyte subsets among healthy blood donors in Hong Kong by a single-platform method, lyse-no-wash flow cytometry (1). The percentage and absolute count of $CD3^+$ $CD4^+$ T helper cells,

TABLE 3 Comparison of lymphocyte subsets between the age groups <40 and ≥40 years of age

Lymphocyte subset	Result for group:		
	<40 yr of age (n = 155 [69 males, 86 females])	≥40 yr of age (n = 118 [81 males, 37 females])	P value[a]
No. of $CD3^+$ cells/μl (%)	744–2,369 (54.72–84.50)	659–2,093 (56.26–84.06)	0.004* (0.003*)
No. of $CD4^+$ cells/μl (%)	404–1,263 (27.41–51.78)	395–1,396 (30.07–55.72)	0.706 (0.019*)
No. of $CD8^+$ cells/μl (%)	267–1,065 (18.15–41.19)	218–926 (14.60–43.46)	0.000* (0.000*)
CD4/CD8 cell ratio	0.70–2.56	0.71–3.57	0.000*
No. of $CD19^+$ cells/μl (%)	127–644 (7.08–26.12)	96–683 (7.34–25.72)	0.640 (0.476)
No. of $CD56^+$ cells/μl (%)	57–611 (3.67–25.70)	86–607 (3.40–28.78)	0.473 (0.015*)

[a] *, statistically significant at $P < 0.05$.

and hence the CD4/CD8 cell ratio, were significantly higher in the present study, while the absolute count of $CD56^+$ NK cells was lower than that reported previously in the Hong Kong Chinese population (12) (Table 1). The differences may be attributed partly to an improvement of methodology as dual-platform flow cytometry was employed in the previous study and partly to the continuous improvement in nutrition and hygiene in our population. A comparison between the present study and the published data from different ethnic groups was also made (Table 2): our results were comparable to those of the Chinese population established in other parts of China (10, 11) and were also similar to those in other Asian populations (8, 17). The reference ranges, however, were different from those reported in Caucasian countries (9) and the limited African data (13).

Sex influences on lymphocyte subset constitution have also been addressed in various ethnic groups (5, 9, 10, 15, 17, 18). Our study also has a higher absolute count and percentage of $CD3^+$ T cells in females compared with males, while the absolute count and percentage of $CD56^+$ NK cells are higher in males ($P < 0.05$). This is probably due to different hormonal effects in the respective genders. Androgen has been shown to accelerate apoptosis of thymocytes and mediate the process of thymocyte selection (19). The relatively low androgen level in females may account for a slower apoptotic rate in T cells. $CD56^+$ NK cells have also been shown to be higher in the male population in other studies (9, 17), but the cause is obscure.

Age has a consistent impact on lymphocyte subsets and has been demonstrated by numerous studies of various different ethnic groups (5, 8, 9, 11, 12, 14, 17, 18). Such an observation has also been reproduced in the present study. We have shown lower values for $CD3^+$ T cells (both percentages and absolute counts), $CD4^+$ T cells (percentages only), and $CD8^+$ T cells (both percentages and absolute counts) in the older age group (≥40 years old). Stem cell aging and thymic involution are implied to contribute to the reduction of T cells in the elderly (9). T cell immunosenescence may play a role in increased morbidity and mortality with age. On the contrary, the percentage of $CD56^+$ NK cells is higher in the older age group, which is probably related to cumulative exposure to infections as one gets older.

Racial differences in the distributions of various lymphocyte subpopulations have been addressed in many studies (5, 9, 10, 14, 15). Table 2 summarizes a comparison of the reference ranges of lymphocyte subsets among Asians, Caucasians, and Africans. The absolute counts of $CD3^+$ T cells and $CD3^+$ $CD4^+$ T helper cells and the CD4/CD8 cell ratio in Hong Kong Chinese are comparable to those of Asians of other countries and Africans but lower than those of Caucasians. The absolute count of $CD3^+$ $CD8^+$ T suppressor cells is similar to that of other Asian populations but higher than those of Caucasians and Africans. The absolute count and percentage of $CD56^+$ NK cells in Hong Kong Chinese are lower than those of other Asian populations but comparable to those of Caucasians. Our study thus further reinforces the importance of establishing local reference ranges in order to assist clinicians to make meaningful clinical decisions. The thresholds for CD4 count for the initiation of antiretroviral therapy and prophylactic antibiotics for the treatment of HIV and AIDS are based on Caucasian studies. In view of the significant differences in the reference ranges in lymphocyte subsets between different ethnic groups, in particular the absolute count of $CD3^+$ $CD4^+$ T helper cells in Chinese, which has been shown to be lower than that of Caucasians (8, 11, 12) (Table 2), the thresholds for the initiation of such therapies should be adjusted accordingly. In fact, it has been suggested that even the thresholds for CD4 count in the definition of AIDS should be different among different racial groups (11). The underlying reasons for the observed variations have not been exactly pinpointed, but differences in genetic makeup and variations in environmental exposure, including diet and microbial challenge, may be part of the attributing factors.

In this study, we have established local reference ranges for lymphocyte subsets for healthy Chinese adults. These are the first reference ranges for Chinese adults established by a single-platform flow cytometric analysis. Age, gender, and ethnicity are major factors contributing to the variations in lymphocyte subset composition. It is recommended that each laboratory should establish its own reference ranges to facilitate clinical decision making.

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