Lymphocyte Subset Reference Ranges in Healthy Israeli Adults

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ABSTRACT: **Background:** Reference ranges for adult peripheral blood lymphocyte subsets have been established in a few countries. To the best of our knowledge no broad lymphocyte subset analysis of the Israeli population has been reported.

Objectives: To establish reference ranges for healthy adults in Israel and to describe age- and gender-specific differences, if present.

Methods: Lymphocyte subsets CD3, CD3/CD4, CD3/CD8, CD3-/CD16+/CD56+, CD3/TCRαβ, CD3/TCRγδ, and CD19 were examined by flow cytometry in 326 subjects. Samples were subdivided according to age and gender.

Results: Women of all ages had a significantly higher percentage and absolute counts of CD3/CD4 cells than their male counterparts. Higher CD3/CD4 cells were observed also in the older population (› 50 years). CD3/CD8 and CD3-/CD16+/CD56+ were higher in males. Older males had a lower total lymphocyte percentage and CD19 cells compared to younger men. No significant gender-related differences were observed in percent and number of CD19, CD3/TCRαβ or CD3/TCRγδ at all ages.

Conclusions: These reference values could be useful in further studies for assessing changes that occur in different populations in human pathology.

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Reference ranges for adult peripheral blood lymphocyte subsets have been established in a few countries. Most studies were restricted to younger adults for monitoring human immunodeficiency virus (HIV) infections. Immunophenotyping of peripheral blood lymphocytes by flow cytometry is routinely performed as part of the diagnosis and management of congenital and acquired immune deficiency syndromes, leukemia and lymphoma. Ethnic origin, age and gender-related changes in blood lymphocyte subpopulations among healthy adults have been reported in several studies [1-3], with some demonstrating contradictory findings regarding the influence of age on the distribution of CD4+ and CD8+ T cells [2,4-7]. These differences can be attributed in part to various factors, such as ethnic origin, genetics, environment, age and gender.

Israeli society is ethnically very diverse with a high incidence of mixed marriages. To the best of our knowledge, broad normal range studies in adults in Israel have not been conducted. In this monocentric study we analyzed the relative and absolute number of lymphocyte subsets in healthy adults by gender and over a wide age range to establish reference values which can be used not only for HIV monitoring but also for a variety of disease states. Significant differences in lymphocyte subsets in different age and gender groups were compared to similar data in the literature.

MATERIALS AND METHODS

The study group samples were from remnants of clinical samples that were taken for complete blood counts from the personnel clinics of Beilinson Hospital in Petah Tikva (n=62) and samples that were taken from normal controls during the last 10 years (n=264), used for rare testing such as for PNH (paroxysmal nocturnal hemoglobinuria) that requires comparison to a normal sample. These data were collected to establish normal range determination for the flow cytometer laboratory. The use of these data from these anonymous subjects for publication was approved by the local Helsinki Committee of Beilinson Hospital.

VARIABLES STUDIED

The following variables were recorded: age, gender, and complete blood count. Percent and absolute counts of the various lymphocyte populations were collected from the lymphocyte gate as follows: T lymphocytes: total CD 3, CD3/CD4, CD3/CD8, CD3/TCRαβ, CD3/TCRγδ, natural killer (NK) cells CD3-/CD16+/CD56+, and B lymphocytes: CD19.

METHODS OF ANALYTIC DETERMINATION

Peripheral blood samples obtained by venipuncture were collected in K3-EDTA anticoagulant and processed within 3 hours of collection. Blood cell counts were performed on

Table 1. Combination of antibodies used

Fluoro chrome	FITC (fluorescein isothiocyanate)	PE (phycoerythrin)	ECD (PE-Texas red)	PC5 (phycoerythrin cyanin 5.1)	PC5.5 (R-phycoerythrin cyanin 5)	PC7 (R-phycoerythrin cyanin 7)	APC (allophyco-cyanin)
Tube 1	CD16 3G8	CD56 N901 (NKH-1)	CD19 J3-119		CD3 UCHT1	CD4 SFCI12T4D11	CD8 B9.11
Tube 2	CD4-CD8 SFCI12T4D11-SFCI21Thy2D3		CD19 J3-119	CD3 UCHT1		CD45 J.33	
Tube 3	TCRαβ IP26	TCRγδ IMMU510	CD3 UCHT1	CD45 J.33			

an automated hematology analyzer (Advia 2120i, Siemens, Germany). Anticoagulated venous blood was aliquoted in 100 ul amounts into 12.75 mm polypropylene tubes (Beckman Coulter, USA) and incubated in the dark for 15 minutes at room temperature with the appropriate fluorochrome-conjugated monoclonal 4–6 antibody combinations at the manufacturer's recommended concentration. Antibody combinations and clones are listed in Table 1. All antibodies were purchased from Beckman Coulter except for TCRαβ, which was purchased from Biolegend (USA). Some samples were repeated with the antibody combination of CD4FITC-CD8PE from Dako (Denmark). Stained whole blood samples were lysed and fixed with the Immunoprep reagent system using the TQ-Prep Workstation (Beckman Coulter). Fluorescence analysis was performed using a Beckman Coulter Navios multiparameter flow cytometer and later analyzed with Kalusa or Navios Software (Beckman Coulter, USA). A minimum of 30,000 events was collected. Absolute counts of circulating cell subsets were calculated using the percentages obtained by flow cytometry and the leukocyte count obtained from the hematological analyzer. A range of internal quality assurance procedures were followed, including daily calibration of flow cytometer optical alignment and fluidic stability using Flow-Check (Beckman Coulter) fluorospheres.

STATISTICAL ANALYSIS

The assumption of a normal distribution for continuous variables was verified using graphic examination of histograms and the Kolmogorov-Smirnov test. When the assumption of normality was valid, the Student *t*-test (for comparison between two groups) or ANOVA tests, with Tukey's correction for multiple comparisons (for more than two groups) were used to compare the mean values of the quantitative variables. When the hypothesis of normality for the quantitative variable was not accepted, the non-parametric Mann-Whitney U test was used. Significance was defined as a *P* value less than 0.05. Data were analyzed using the statistical software SAS, version 9.4.

RESULTS

DEMOGRAPHIC PARAMETERS

The study population included 326 subjects: 62 with an average age of 46 (range 28–78 years) were recruited from staff clinics of the Beilinson Hospital in Petah Tikva, and 264 subjects with an average age of 55 years (range 17–96) who served as normal controls. There were no significant statistical differences between these two groups with regard to flow cytometric data (data not shown). Age-dependent studies were conducted as follows: subjects were divided into two groups: "young adults" until the age of 49 (n=114) and "older adults" of 50 years and above (n=212).

LYMPHOCYTE SUBSETS

The percentage and absolute number of peripheral blood total, helper, and cytotoxic T cells were estimated, based on the expression of CD3, CD4, and CD8, respectively. (Double-positive CD4/CD8 was less than 2% in all samples). The percentage and absolute mean values of the lymphocyte subsets are shown in Table 2. The mean values are grouped according to gender in Table 3.

Table 2. Peripheral blood lymphocyte subsets (as % of total lymphocytes)

N = 326	Minimum	Maximum	Average	Median
Age, yrs	17	94	56.7 ± 17.2	59
Lymphocytes,%	19	48	28.43 ± 6.04	27.7
WBC, cells/μl	4.8	10.9	6.92 ± 1.41	6.67
T lymphocytes as % of total lymphocytes				
%CD3	34	98	73.39 ± 9.6	74
%CD/3CD4	10	76	45.39 ± 56	45
%CD3/CD8	6	65	29.91 ± 8.1	29.5
%CD3-/CD16/CD56	3	31	9.82 ± 5.05	9
CD3/TCRαβ (n=50)	37	97	64.19 ± 18.38	
CD3/TCRγδ (n=50)	0	14	2.2 ± 3.29	
B lymphocytes as % of total lymphocytes				
CD19	2	53	12.25 ± 7.5	11
T lymphocyte, cells/μl				
Abs-CD3	433.7	4083	1446.4 ± 89	1380
Abs-CD3/CD4	65	3999	888.5 ± 415.5	821.7
Abs-CD3/CD8	41	2914	591.4 ± 330.7	528.8
CD3-/CD16/CD56 Abs	52	547	193.2 ± 102	171
B lymphocytes, cells/μl				
Abs-CD19	12	1010	240 ± 162	206

There was no significant difference regarding average age and percentage of lymphocytes between men and women. Both percentage and absolute cell count of CD3/CD4 were significantly higher in women, while the percentage and absolute cell number of CD3/CD8 and CD3-CD16+CD56+ were significantly higher in men [Table 3]. No significant difference was observed in CD19, CD3, CD3/TCRαβ, CD3/TCRγδ in both percentage and number of cells (cells/μl)

With regard to age, lower lymphocyte percentages were observed in the older group compared to the young adults and was significant only in men [Table 4]. Both percentage and absolute cell count of CD3/CD4 were significantly higher in the older group of both sexes. However, the CD3/CD4 population remained significantly higher in females of both age groups as compared to males. A significant lower percentage and absolute cell number of CD3/CD8 were observed only in the older group of women compared to younger women and to older men. A lower percentage and absolute cell number of CD19 levels were observed in the older male group compared to the younger men and also between young men and women. No significant difference was observed between the sexes in the older group. The levels of CD3-CD16+CD56+ were significantly higher in men in both age groups

Table 3. Peripheral blood lymphocyte subsets by gender

	Female N=152			Male N=174			*P* value
	Min	Max	Average	Min	Max	Average	
Age, yrs	17	92	56.7 ± 17.28 (58.5)	18	94	57.13 ± 16.9 (59)	NS
Lymphocytes, %	19	48	29.02 ± 6.6 (28)	19	46	27.97 ± 5.53 (27.7)	NS
WBC, cells/μl	4.8	10.9	6.71 ± 1.46 (6.4)	4.8	10.7	7.03 ± 1.38 (6.8)	0.0431
T&NK lymphocytes, %							
%CD3	45	97	74.55 ± 9.62 (75)	34	98	72.5 ± 9.55 (74)	NS
%CD3/CD4	10	70	49.33 ± 13.03 (48)	10	76	42.4 ± 13.36 (41)	0.02
%CD/3CD8	2	70	26.83 ± 10.6 (26)	2	80	32.25 ± 12.5 (32)	0.0011
%CD3-16+56+	3	20	8.03 ± 6.16 (6)	6	31	12.3 ± 4.13 (11)	0.0008
CD3/TCR-αβ	16	97	65.8 ± 21.1	37	86	63.7 ± 18.1	NS
%CD3/TCR-γδ	0	13	2.25 ± 3.47	0	14	2.2 ± 3.3	NS
T lymphocytes, cells/μl							
CD3	648	4083	1466.9 ± 537.2 (1393)	433.7	3060.3	1430 ± 450.4 (1380)	NS
CD3/CD4	197	8999	964 ± 469 (892)	67	2652.1	830.67 ± 360 (775)	0.0012
CD3/CD8	96	1290	529 ± 244 (483)	154	1539	613 ± 242 (586)	0.0002
CD3-16+56+	52	315	153 ± 112 (140)	99	547	44 ± 87.4 (237.8)	0.0004
B lymphocytes							
B lymph, %CD19	2	39	12.18 ± 7.94 (10.5)	2	53	12.35 ± 7.05 (11)	NS
B lymph CD19, cells/μl	12	1010	241 ± 182 (185)	31	762	239.1 ± 145 (211)	NS

DISCUSSION

This study assessed a variety of lymphocyte subsets, including CD3, CD3/CD4, CD3/CD8, CD3-/CD16+/CD56+, CD19, CD3/TCRαβ, CD3/TCRγδ, in the Israeli population. Borkow et al. [8] had compared the CD4/CD8 ratio and CD4 and CD8 absolute cell number in 34 normal non-Ethiopian origin Israeli immigrants to Ethiopian immigrants. Although their control group was small, the average absolute CD3, CD4 and CD8 (1560 ± 63 cells/μl, 853 ± 44 cells/μl and 638 ± 34 cells/μl respectively) were similar to ours (1486 ± 243, 1422 ± 426: 868 ± 146, 882±270 cells/μl and 604 ± 172, 574 ± 238 cells/μl respectively) [Tables 2 and 3]. Comparison of our results to studies with normal ranges in several countries – such as Germany [2,3], Switzerland [5], Iran [4], India [7], Italy [6], Spain [9], West Africa [10], Ethiopia [11] and England [12] – have shown that the range and mean CD3 CD4 CD8 percentages and absolute counts were similar to ours in most of the studies. Recent data from 268 healthy adults in China showed a lower percentage of CD4 compared to our data [13].

An association of aging with evidence of immune deficiency and dysregulation was previously suggested. However, in our study, both percentage and absolute cell count of CD3/CD4 were significantly higher in the older group. Gregg et al. [14] had demonstrated an increase in peripheral blood CD4 +CD25 high regulatory T cells associated with aging but did not provide data on the total CD3/CD4 population. Our results also show lower CD3/CD8 percentage and cell number in the older female group and significantly lower percentage and absolute cell number of CD19 in older men. The levels of CD3-CD16+CD56+ were significantly higher in men in both age groups, reflecting gender-only specificity.

There are contradictory reports in the literature with reference to age: different studies observed a decrease in both CD4 and CD8 T lymphocyte subsets and CD19+ B cells with age [3,5,15], a decrease in CD3 and CD8 but not in CD4, a decrease in CD3+/CD8+ T cells with increased counts of NK cells [2], or no significant changes at all [6,7,16]. Melzer and colleagues [3] found decreased cytotoxic T cells with age. A paper from China [13] demonstrated an decrease in CD8+ cell counts and increase in CD4+ cell percentages and counts with age in adults between 21 and 60 years of age. The contradictory results in the literature may reflect the different age range and age grouping strategies. When we divided the groups into 10 year intervals, not all changes could be demonstrated.

Gender-related studies have shown that both percentage and absolute cell CD3/CD4 cells/μl were significantly higher in women, while the percentage and absolute cell number of CD3/CD8 were higher in men. NK cells (CD3-/CD16+/CD56+) percentage and absolute number (cells/μl) were also significantly higher in men. Higher values of CD3 and CD3/CD4 T cells and lower values of NK cells in women was previously described in a population of healthy Italian adults [7].

Table 4. Peripheral blood lymphocyte subsets by age group

	Total average			Men average			Women average			Men:Women *P* value	
Age (yrs) N	**< 49 114**	**≥ 50 212**	***P* value**	**< 49 61**	**≥ 50 124**	***P* value**	**< 49 53**	**≥ 50 88**	***P* value**	**< 49 61:53**	**≥ 50 124:88**
WBC, cells/µl	6.7 ± 1.3 (6.3)	7.03 ± 1.4 (6.85)	0.04	6.69 ± 1.25 (6.7)	7.2 ± 1.4 (7.2)	0.017	6.7 ± 1.5 (6.37)	6.85 ± 1.46 (6.35)	NS	NS	0.044
Lymphocytes, %	29.4 ± 5.5 (29)	27.9 ± 6.2 (27)	0.03	29.46 ± 5.3 (29)	27.24 ± 5.5 (27)	0.01	29.4 ± 4.9(28.9)	28.8 ± 7.1 (27.3)	NS	NS	NS
T&NK lymphocytes, %											
%CD3	73.8 ± 8.5 (75)	73.2 ± 10.2 (74)	NS	71.8 ± 8.4 (74)	72.8 ± 10.1 (74)	NS	76 ± 8.1 (77)	73.6 ± 10.4 (72.5)	NS	0.008	NS
%CD3/ CD4	42.37 ± 9 (42)	47.01 ± 15.3 (46)	0.0007	39.5 ± 7.64 (40)	43.8 ± 15.2 (43.5)	0.01	45.6 ± 9.5 (47)	51.5 ± 14.3 (50)	0.054	< 0.001	< 0.001
%CD3/CD8	30.9 ± 8.9 (28)	29.4 ± 13 (30)	NS	31.4 ± 7.95 (32)	32.67 ± 13.8 (32)	NS	30.26 ± 9.9 (27)	24.7 ± 10.5 (25)	0.0023	NS	< 0.001
%CD3-CD16+/56+	9.33 ± 4 (9)	11.27 ± 7.3 (9)	NS	11.05 ±3.92 (10)	15.33 ± 8.5 (13)	0.021	7.83 ± 3.6 (6)	8.56 ± 5.25 (6)	NS	0.006	NS
T&NK lymphocytes, cells/µl											
CD3	1437 ± 366 (1420)	1450 ± 544 (1374)	NS	1400 ± 341 (1323)	1445 ± 496 (1382)	NS	1481 ± 393 (1437)	1458 ± 610 (1296)	NS	NS	NS
CD3/CD4	824 ± 246 (821.8)	923 ± 479 (821.7)	0.0148	771 ± 222 (759.7)	860 ± 409 (792.6)	0.058	885 ± 260 (897.5)	1012 ± 554 (884.9)	NS	0.013	0.03
CD3/CD8	603 ± 233 (552)	585.2 ± 373.2 (516.4)	NS	610 ± 197 (588)	654 ± 400 (582)	NS	594 ± 270 (529)	488 ± 309 (428)	0.04	NS	< 0.001
CD3-CD16+/56+	190.5 ± 94.7 (170.9)	201.2 ± 127 (192.8)	NS	230 ± 100 (237.8)	282 ± 152 (250.6)	NS	155 ± 75 (141)	147 ± 76 (116)	NS	0.006	0.04
B lymphocytes											
B lymphocytes %CD19	13.6 ± 7.33 (13)	11.8 ± 7.4 (10)	NS	15.05 ± 6.46 (15)	11.44 ± 7.03 (10)	0.04	11.55 ± 8.1 (9)	12.41 ± 7.9 (11)	NS	0.05	NS
B lymphocytes CD19, cells/µl	258 ± 146 (247)	233.6 ± 167.6 (187)	NS	285.6 ± 143 (264.3)	223 ± 146 (191.7)	0.021	219 ± 143 (218)	249 ± 195 (184)	NS	NS	NS

Median appears in parentheses. Mean ± SD is shown
NS = not significant

Similar results were obtained in India [7], England [12], West Africa [10] and Germany [2,3]. Andreu-Ballester et al. [9] reported similar results in a Spanish control group, although in their study CD3 percentage and absolute number (cells/µl) were significantly higher in women. Total T cell percentages were higher in females from a Chinese population [13].

In our study there was no difference in the total CD3 percentage or CD3 count. It is tempting to consider that hormonal differences, therapy or menopause could be related to the differences between men and women. Several studies indicate that sex hormones modulate immunity, but the impact of menopause and hormone replacement therapy on immune function remains poorly understood [17].

Ovarian steroids can modulate T and B cell function directly through binding of sex steroid receptors such as estrogen receptors (ER) expressed by lymphocytes. Different cells express different receptors. CD4 T cells express a higher amount of ERα than ERβ, B cells express more ERβ than ERα. CD8 T cells express only low amounts of both receptor types [17,18]. It is possible that the expression of different hormone receptors on T lymphocytes may contribute to the differential cell proliferation in females as compared to men.

Our investigation has several limitations: the influence of environmental factors, such as smoking, diet and lifestyle were not taken into account, and neither was ethnic diversity. This information would not be available to us in our test population. Serological testing for chronic disease or infections was not performed and a vague possibility remains that some diseases may have been overlooked.

In conclusion, we have established a normal reference range in Israel for lymphocyte subsets. The most pronounced differences were gender related. The percentage and absolute cell number of CD3/CD4 were significantly higher in women, while the percentage and absolute cell number of both CD3/CD8 and CD3-/CD16+/CD56+ were higher in men. We believe that these reference values are potentially useful in diagnostic hematology and subsequent research studies for assessing changes that may occur in human pathology.

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