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Determination of lymphocyte subset reference ranges in peripheral blood of healthy adults by a dual-platform flow cytometry method

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ABSTRACT

Flow cytometry has emerged as a useful screening approach to evaluate whether specific cell populations are present or absent. Previous studies have shown different reference ranges in several countries. The aim of this study was to determine reference ranges of lymphocyte subsets in peripheral blood by flow cytometric method in Brazilian adults. In this study, relative and absolute reference ranges of lymphocyte subsets were: CD3+: 51.3–83.5%, 718–2494 cells/μl; CD4+: 24.4–54.2%, 456–1492 cells/μl; CD8+: 12.8–40.2%, 272–1144 cells/μl; CD4+CD8+: double-positive 0.01–3.6%, 2–88 cells/μl; TCR γδ: 1.0–15.9%, 19–345 cells/μl; CD3+CD4−CD8−: 1.2–13.3%, 28–292 cells/μl; TCR αβ+: 44.3–77.0%, 855–2384 cells/μl; CD4/CD8 ratio: 0.68–3.61; CD19+: 6.3–20.8%, 112–622 cells/μl; mature NK cells: 3.1–27.4%, 70–745 cells/μl; immature NK cells: 0.08–1.1%, 1–23 cells/μl; total NK cells: 3.7–28.5%, 82–760 cells/μl; and NKT cells: 0.9–21.4%, 18–488 cells/μl. Comparison with other studies showed differences among some of them. This suggests that there are differences among lymphocyte subsets in the worldwide population and also it is important to determine reference ranges in different populations in order to better assess and monitor patients.

1. Introduction

The use of flow cytometry in clinical laboratory has grown substantially in the past decade. This method has emerged as a useful screening approach to evaluate whether specific cell populations and subpopulations are present or absent, which has been useful to clarify or assist diagnosis of leukemias, lymphomas, and immunodeficiencies [1]. For diagnosis purposes and/or treatment decisions, flow cytometry results should be compared with a reference interval [2–4] and, due to the characteristics of different population, it is recommended that each laboratory establish their own reference range [2,5]. Unfortunately, few laboratories do so, thus most patients have their results compared with reference intervals obtained from populations that do not represent them. Immunophenotyping by flow cytometry is widely used for relative and absolute quantification of white blood cells and, therefore, it is required that laboratories establish reference ranges for relative and absolute lymphocyte subsets in peripheral blood samples [6]. Many studies have shown that different populations have distinct reference ranges [4,6–22]. For example, Pasqualetti et al. [18] demonstrated that individuals from the Philippines have higher relative and absolute NK cell counts than Italians. This large difference was attributed to genetic factors, since subjects living in the same geographic region were expected to be exposed to the same type of antigens, thus minimizing this kind of influence. In Brazil, one study [23] has evaluated relative and absolute lymphocyte counts and reference ranges for healthy subjects. Therefore, the aim of

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the present study was to determine reference ranges of lymphocyte subsets in peripheral blood by a dual-platform flow cytometry method in healthy Brazilian adults.

2. Materials and methods

2.1. Subjects

A total of 238 adult non-smoker blood donors – 134 men (56.3%) and 104 women (43.7%) – with mean age of 27.3 ± 9.3 years; range: 16–56 years, were randomly selected from the Hemotherapy Service at the Hospital of the Federal University of Santa Catarina (UFSC). This study was approved by the UFSC Human Research Ethics Committee (registration number 1163/2011). All participants provided their written informed consent before sample collection.

Peripheral blood (PB) samples were collected in EDTA anticoagulant tubes (Vacuette, Greiner Bio-One, Germany) in the morning, from 8 a.m. to 12 p.m., from March 2011 to March 2012. Samples that had clots, hemolysis, or positive serologic test results (HBsAg, anti-HBc, anti-HCV, anti-HTLV-I/II, syphilis, HIV-I/II antigen, and *Trypanosoma cruzi* antibody) were excluded from the study. Previous studies had shown that smokers have a significant alteration in some lymphocyte subset counts [6,24–27], consequently, smokers were not included in this research.

2.2. Processing of peripheral blood samples

Samples were processed by a dual-platform method [8] that used the flow cytometer FACSCanto II (BD Biosciences, USA) for relative value counts and the Sysmex XE-2100D (Sysmex Corporation, Japan) hematology cell analyzer to enumerate absolute lymphocyte counts. Data analysis was performed with the Infinicyt software version 1.6.0 (Cytognos S.L., Salamanca, Spain).

For immunophenotypical analysis, the panel of fluorochrome-conjugated monoclonal antibodies used was: anti-CD4 FITC/anti-CD8 PE/anti-CD3 PerCP/anti-CD45 APC (Tube 1); anti-CD20 FITC/anti-CD19 PE/anti-CD45 PercP (Tube 2); anti-CD16 FITC/anti-CD56 PE/anti-CD3 PerCP/anti-CD45 APC (Tube 3), and TCR αβ FITC/TCR γδ PE/CD3 PerCP/CD45 APC (Tube 4). All monoclonal antibodies were purchased from BD Biosciences, USA, except CD3 and CD45 PerCP, which were obtained from Beckman Coulter, USA. Flow cytometry tests were performed according to the manufacturer's instructions. Immediately after the sample preparation, data was collected using a side-scatter channel (SSC) gate on lymphocyte region versus CD45 dot plot and the count stopped when it reached 10,000 lymphocytes. The threshold was set at 33,000 on the forward-scatter channel (FSC) in order to avoid losing small lymphocytes as much as possible, as their sizes can be very close to that of debris (particles smaller than cells), dead cells, and non-lysed red blood cells.

2.3. Quality control

Instrument setup, calibration, and quality control were performed daily during the study using the commercial standard reagents Cytometer Setup and Tracking Beads (BD Biosciences, USA) for the flow cytometer and e-CHECK (Sysmex Corporation, Japan) for the hematology cell analyzer.

Comparing the relative lymphocyte counts obtained with the flow cytometer and hematology cell analyzer showed no differences when evaluated by *t*-test ($p = 0.926$) and by intraclass correlation coefficient (ICC) ($r = 0.960$).

To verify the reliability of the data obtained by the dual-platform method, 20 PB samples marked with CD3, CD4, and CD8 were analyzed. A comparison was established between results obtained in the flow cytometer FACS Canto II – dual-platform – and data from FACS Calibur (BD Biosciences, USA) – single platform – because the results of the latter are assured by proficiency testing. The ICC of the markers CD3, CD4, and CD8 between the two devices ranged from 0.948 to 0.996 and this correlation was statistically significant between the two methods ($p < 0.0001$) for all variables. These data show that the results obtained by both single- and dual-platform methods were interchangeable.

2.4. Statistical analysis

Statistical analysis was carried out using the software SPSS (version 17.0, USA). Reference ranges were calculated using mean ± 2 standard deviation for parametric data and 2.5% and 97.5% percentiles for non-parametric data. In all cases, differences were considered statistically significant when $p \leq 0.05$. Genders were compared using *t*-test for parametric data and Mann–Whitney test for non-parametric data.

3. Results

The lymphocyte subsets evaluated in this study were: T CD3+, T CD4+, T CD8+, double-positive T CD4+CD8+, T TCR γδ+, T CD3+CD4−CD8−, CD4/CD8 ratio, B CD19+CD20+, B CD19+CD20−, NK CD56+CD16+ (mature NK cells), NK CD56++CD16− (immature NK cells), total NK cells, and NKT cells.

Relative and absolute counts were determined for lymphocyte subsets in the overall population, as well as for males and females (Table 1). The comparison of the present data with that of other studies can be seen in Table 2.

Results of gender comparison showed statistically significant differences for T CD3+, T CD4+, double-positive T CD4+CD8+, negative B CD19+CD20−, and mature and total NK cells for relative and absolute counts. T CD3+, T CD4+, double-positive T CD4+CD8+, and B CD19+CD20− had higher values for females, while mature and total NK were higher for males. For T CD8+, significant differences were found only for absolute value and females also had higher counts. For T TCR γδ+ cells, CD4/CD8 ratio, T CD3+CD4−CD8−, B CD19+CD20+, and immature NK cells, the difference was not significant.

Because double-negative T CD4−CD8− cells express TCR γδ heterodimers on their surface, these cells were also evaluated for phenotype CD3+CD4−CD8−. The T CD3+CD4−CD8− counts obtained from Tube 1 had a good correlation with TCR γδ+ counts obtained from Tube 4 (ICC: $r_{relative} = 0.787$ and $r_{absolute} = 0.813$). This also occurred when relative (female $r = 0.860$ and male $r = 0.753$) and absolute (female $r = 0.881$ and male $r = 0.783$) values were divided by gender.

The number of positive cells for CD19 and negative cells for CD20 was also assessed and, finally, it was verified whether the B cells were positive for other markers. As a result, they were CD38++, CD27++, and CD45++; and lacked CD56 and CD138 expression.

4. Discussion

Results obtained in this study were difficult to compare with others because there was no uniformity in the way other analyses expressed their data. Some studies only showed absolute values for the overall population [12,13,17], while other investigations just showed values split by gender (without the number of total samples) [9,15,16,20,21]. In addition, there were studies that did not calculate the reference range, showing only the mean or median values [7,18]. Furthermore, the population of all studies differed in age, ethnical, and environmental characteristics [6,8].

Table 1
Lymphocyte subsets (median and reference ranges) of 238 adult blood donors in total and split by gender.

	Total (n = 238)		Male (n = 134)		Female (n = 104)		Gender comparison
	Median	Reference range	Median	Reference range	Median	Reference range	p
T CD3+							
%	72.5	51.3–83.5	70.8	48.0–82.6	75.1	56.8–84.1	<0.0001*
cells/μl	1557	718–2494	1463	735–2271	1662	1259–2702	<0.0001*
T CD4+							
%	39.5	24.4–54.2	37.8	23.0–52.6	40.7	26.9–55.5	<0.0001*
cells/μl	844	456–1492	778	427–1372	922	538–1850	<0.0001*
T CD8+							
%	26.5	12.8–40.2	25.1	12.6–39.2	27.2	13.3–41.5	0.100
cells/μl	555	272–1144	535	243–1068	580	291–1238	0.013*
CD4+CD8+							
%	0.5	0.01–3.6	0.5	0.06–3.4	0.6	0.1–4.1	0.038*
cells/μl	12	2.0–88	10	2.0–86.0	13	3.0–89	0.009*
TCR γδ+							
%	4.4	1.0–15.9	4.3	0.8–16.6	4.6	0.9–14.6	0.906
cells/μl	94	19–345	91	17–355	96	21–349	0.405
CD3+CD4−CD8−							
%	4.1	1.2–13.3	4.0	1.1–14.6	4.2	1.2–11.4	0.345
cells/μl	89	28–292	87	21–196	94	31–282	0.937
CD4/CD8 ratio	1.52	0.68–3.61	1.50	0.69–3.58	1.53	0.61–3.79	0.547
B CD19+CD20+							
%	11.5	6.3–20.8	11.2	6.1–21.6	12.0	5.9–20.6	0.309
cells/μl	252	110–618	223	101–585	259	113–658	0.060
B CD19+ CD20−							
%	0.1	0–1.3	0.1	0.004–1.3	0.2	0.008–1.4	0.026*
cells/μl	3	0–23	2	0–26	4	0–31	0.002*
Mature NK cells							
%	10.5	3.1–27.4	12.3	4.3–31.2	8.2	2.8–24.1	<0.0001*
cells/μl	222	70–745	254	92–806	201	60–663	<0.0001*
Immature NK cells							
%	0.32	0.08–1.1	0.30	0.08–1.1	0.36	0.04–1.2	0.798
cells/μl	8	1–23	8	1–20	9	1–25	0.247
Total NK cells							
%	11.4	3.7–28.5	13.0	5.0–31.3	9.1	3.5–24.9	<0.0001*
cells/μl	234	82–760	273	111–829	218	65–689	<0.0001*
NKT cells							
%	6.0	0.9–21.4	5.8	0.9–19.4	6.1	0.5–21.5	0.214
cells/μl	134	18–488	127	20–436	139	12–610	0.053

* Statistically significant ($p \leq 0.05$).

The T CD3+ relative reference range found in this study was similar to those reported in several studies [4,6,11,14–17,19] (Table 2). However, when absolute values were compared, some differences were found between populations. In the Indian population, for example, the range was wider than in this study. One explanation for the difference might be that India is a heterogeneous country and four geographical areas of the country had their samples evaluated in the study [19]. The Brazilian population is also heterogeneous, having an ethnic diversity in each region of the country as a result of different colonization, since immigrants who arrived in Brazil between 1500 and 1972 were 58% Europeans, 40% Africans, and 2% Asians [28,29]. Regardless of that, the population in this study was homogeneous (97.9% Caucasians) since the evaluation was carried out in only one state located in southern Brazil which was colonized mainly by Europeans. Similarly to the Indian study, another research conducted in Brazil showed reference ranges wider than the ones found in this investigation. This can be explained by the fact that it evaluated two different states (Bahia and Pará) with distinct colonization (African descendents and native Amerindians, respectively). These differences can be justified by methodological differences and environmental factors [23]. Unlike results in this study, most of the results showed no difference in T CD3+ relative and absolute values between genders [4,9,11,14,20]. Nevertheless, this study is in agreement with the values reported in previous studies [3,6,19,22] that observed the presence of higher T CD3+ lymphocyte values in females.

Relative and absolute reference ranges for T CD4+ cell count calculated in this study were similar to other ones (Table 2), except for the Indian population, which had values with a wider range [19]. Moreover, relative and absolute values for T CD4+ count were higher in females and these findings were consistent with previous studies [3,6,7,14,16,17,19,22,23]. Maini and colleagues suggested that hormonal differences between genders might explain this difference in T CD4+ counts [15]. Comparing this study with another one conducted in Brazil [23] showed differences in T CD4+ absolute reference range.

As shown in Table 2, T CD8+ relative and absolute counts were comparable to values reported in other studies [4,6,9–11,14,21,22], except for a German study which reported a narrower range [11]. Most studies showed no difference between genders [4,6,7,9,11,13,14,21,22]. In this study, gender comparison showed differences only for T CD8+ absolute count, for which female had higher values, similarly to those reported in adults in the northern rural region of Tanzania [16]. Comparing the results of this study (Table 1) with those by Torres et al. [23] showed differences in T CD8+ reference ranges for healthy Brazilian people.

Double-positive T CD4+CD8+ cells were a poorly characterized population of effector T cells and can be found in peripheral blood at low frequencies [30,31]. Chauhan et al. [30] observed that the range for their uninfected control group (n = 10) was 0.1–1.2%. However, in this study, the calculated range for healthy adults was wider, probably because a larger number of persons were evaluated.

Regarding the reference range determination of T TCR γδ+ cells, differences between males and females were not significant. Moreover, results showed that TCR γδ+ values can be obtained using the double-negative CD3+CD4−CD8− phenotype.

Table 2
Comparison among studies of lymphocytes relative and absolute reference ranges.

T CD3+ (%)	T CD3+ (cells/μl)	Reference	T CD4+ (%)	T CD4+ (cells/μl)	Reference
52.30–84.64	725–2960	[4]	30.0–60.34	437–2072	[4]
60.0–87.0	605–2460	[6]	32.0–61.0	493–1666	[6]
49.0–80.0	796–2679	[9]	23.0–48.2	401–1451	[9]
53.0–80.0	988–3912	[10]	19.0–46.0	431–1976	[10]
53.0–83.0	780–2240	[11]	30.0–59.0	490–1640	[11]
57.0–80.0	1069–2921	[14]	30.0–53.0	631–1696	[14]
55.0–81.0	457–3926	[19]	14.0–60.0	302–2371	[19]
53.7–82.6	769–2798	[22]	27.0–55.0	405–1500	[22]
51.3–83.5	935–2572	Present study	23.5–55.7	456–1492	Present study

T CD8+ (%)	T CD8+ (cells/μl)	Reference	CD4/CD8 ratio	Reference
17.76–39.94	307–1184	[4]	1.06–2.76	[4]
14.0–43.0	224–1112	[6]	0.69–2.83	[9]
13.4–41.0	243–1206	[9]	0.90–5.00	[11]
17.6–49.0	385–1808	[10]	0.72–2.56	[12]
10.0–40.0	170–880	[11]	0.90–2.80	[14]
16.0–35.0	328–1167	[19]	1.10–2.50	[16]
15.0–41.0	261–1033	[22]	0.70–5.30	[17]
20.0–43.0	218–1396	[21]	0.40–2.40	[20]
14.0–42.9	272–1144	Present study	0.40–3.00	[21]
			0.61–2.73	[22]
			0.69–3.58	Present study

B CD19+ (%)	B CD19+ (cells/μl)	Reference	NK cells (%)	NK cells (cells/μl)	Reference
3.9–20.79	74–586	[4]	0.1–13.2	3.08–367	[4]
5.0–20.0	72–520	[6]	4.0–28.0	73–654	[6]
5.0–22.4	130–716	[10]	6.0–37.0	115–1.009	[9]
5.0–21.0	80–490	[11]	8.0–37.4	227–1354	[10]
6.0–22.0	50–610	[13]	5.0–32.0	80–690	[11]
7.0–22.0	148–658	[14]	4.0–22.0	60–580	[13]
4.0–24.0	56–436	[20]	5.0–29.0	62–549	[20]
6.0–21.0	88–654	[22]	6.0–35.0	118–830	[22]
4.7–19.13	72–460	[36]	5.35–30.93	77–427	[36]
5.1–20.8	82–560	[37]	7.1–38.0	130–938	[37]
5.0–27.0	110–890	[39]	4.0–29.0	90–690	[39]
6.3–20.8	112–622	Present study	3.7–28.5	82–760	Present study

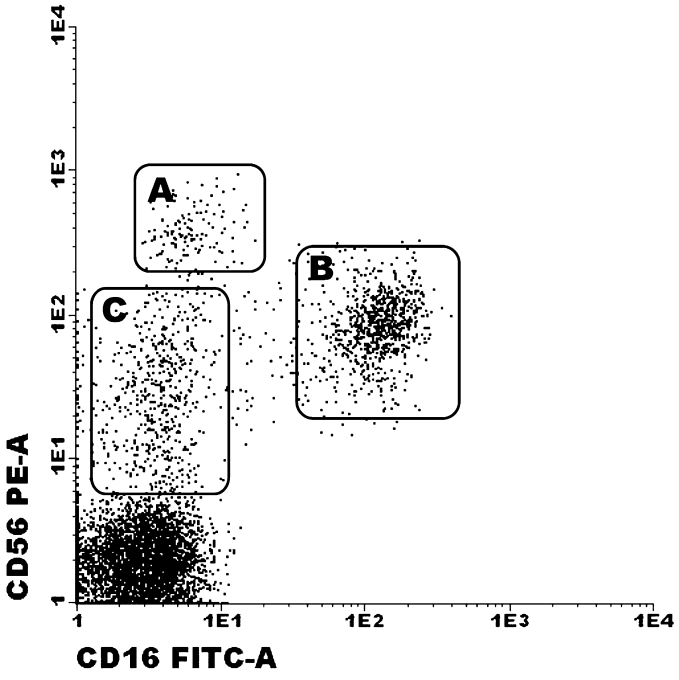

Fig. 1. (A) Representative dot plot of mature NK cell CD56+CD16+; (B) immature NK cell CD56++CD16−; and (C) NKT cells CD56+CD16−.

The CD4/CD8 ratio obtained in this study was comparable with values reported by other studies [4,9,11,12,14,16,17,20–22,30]. However, when gender was evaluated, some studies [11,12,17,21,22] found a higher ratio among females, while others [4,7,9,14,16,32], including this study, did not find differences between genders.

NK cells were characterized by expression of CD16 and CD56 on their membrane [33] and the expression intensity of these markers allowed NK cells to be divided into two subpopulations: immature NK cells (weak or negative CD16 and strong CD56) and mature NK cells (strong CD16 and weak CD56) (Fig. 1A and B) [34,35]. As far as the authors know, no other studies have evaluated the amount of mature and immature NK cells separately. Previous studies have shown different reference ranges for total NK cells in several countries (Table 2) [4,6,9–11,13,18,20,22,35–40]. The NK cell reference range calculated in this study was similar to that reported in research conducted in other countries [6,9–11,13,20,22,35–39], except for the Turkish study that showed a narrower range [4] (Table 2). Kam et al. [37], as well as Yaman et al. [4], found no differences between males and females. However, other investigations were in agreement with results in this study, i.e., differences in relative or absolute counts between genders were observed [6,9,11,22,36].

NKT cells (Fig. 1C) comprise the lymphocyte subpopulation which co-expresses two markers: one associated with NK cells (CD56+) and another associated with the T lineage (CD3+) [33]. Only one other study that calculated the reference range for NKT cells was found, however, it did not assess gender differences and

reported a relative reference range for NKT cells from 0.78% to 11.71% [40].

Previous studies have shown different reference ranges for B cells in several countries – Table 2 [4,6,10,11,13,14,20,22,36–39]. The results in this study were similar to those obtained in other populations [4,10,11,14,22,36]. Unlike these results, most studies found no differences between genders [4,6,11,14,36]. In accordance with results in this study, Urassa et al. [22] found differences between males and females, but unlike this investigation, this difference was found in the relative and absolute counts.

Evaluating the complete phenotype of B CD19+CD20− cells indicated that these cells were early plasmablasts that could exit into peripheral blood and survive for a short period unless they were recruited into mucosa or bone marrow niches, depending on their chemokine receptor expression [41]. No other research that studied this cell population was found to establish comparisons.

5. Conclusions

The lack of standardization in methodology employed and data presentation made it difficult to compare lymphocyte subset results among laboratories in different countries. When results could be compared, differences were observed in some of them. This suggests that there are differences among lymphocyte subsets in the worldwide population and that it is important to determine reference ranges in different populations in order to better assess and monitor patients, especially those with immunodeficiencies. Furthermore, this study found a statistically significant difference in relative and absolute values of some lymphocyte subsets between genders. Therefore, different reference ranges for males and females may have to be considered for some types of cells.

Conflict of interest

None of the authors have any potential financial conflict of interest related to this manuscript.

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