

Reference Values for a Panel of Cytokinergic and Regulatory Lymphocyte Subpopulations

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Lymphocyte subpopulations producing cytokines and exerting regulatory functions represent key immune elements. Given their reciprocal interdependency lymphocyte subpopulations are usually assayed as diagnostic panels, rather than single biomarkers for specialist clinical use. This retrospective analysis on lymphocyte subpopulations, analyzed over the last few years in an outpatient laboratory in Northeast Italy, contributes to the establishment of reference values for several regulatory lymphocytes currently lacking such reference ranges for the general population. Mean values and ranges in a sample of Caucasian patients (mean age 42±8,5 years), were provided for Th1, Th2, Th17, Th-reg, Tc-reg, Tc-CD57⁺ and B1 lymphocytes. The results are consistent with what is found in literature for the single subtypes and are: Th1 157.8±60.3/μl (7.3%±2.9); Th2 118.2±52.2/μl (5.4%±2.5); Th17 221.6±90.2/μl (10.5%±4.4); Th-reg 15.1±10.2/μl (0.7%±0.4); Tc-reg 5.8±4.7/μl (0.3%±0.2); Tc-CD57⁺ 103.7±114.1/μl (4.6%±4.7); B1 33.7±22.8/μl (1.5%±0.9); (Values are mean±SD). The results show that despite their variability, mean values are rather consistent in all age or sex groups and can be used as laboratory internal reference for this regulatory panel. Adding regulatory cells to lymphocyte subpopulations panels allows a more complete view of the state of the subject's immune network balance, thus improving the personalization and the "actionability" of diagnostic data in a systems medicine perspective.

[Immune Network 2016;16(6):344-357]

Keywords: Cytokines, Immune dysregulation, Lymphocyte subsets, Personalized medicine, Reference range, Diagnostic-omics

INTRODUCTION

Lymphocyte subpopulations can be altered in most immune related diseases, and the investigation of such changes can offer an effective means to clearly assess the patient's health status, to monitor the course of a disease, or to evaluate the outcome of treatments (1,2). In

clinical ambulatory practice, lymphocyte subpopulations are analyzed mainly in pathologies directly affecting the immune system, i.e. to monitor immunodeficiencies or lymphoproliferative diseases (3). However, there is growing interest in lymphocyte subpopulations analysis also in pathologies where the immune system is not the main system affected, but its activation or dysregulation

Received on October 26, 2016. Revised on December 3, 2016. Accepted on December 9, 2016.

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Abbreviations: CBC, complete blood cell counts; Tc, T cytotoxic; NEU, neutrophils; LYMPH, lymphocytes; MONO, monocytes; EOS, eosinophils; BASO, basophils

can contribute to the onset or progression of a disease, like in many chronic and degenerative conditions (autoimmune diseases, CNS and peripheral neurodegenerative diseases, etc.) (4-11).

Flow cytometric analysis of lymphocyte phenotypes is normally performed on major lymphocyte groups (12) and a further sub-characterization is usually not considered in the standard lymphocyte phenotyping routine despite known associations between other lymphocyte subsets and certain disease states (13-16). Addition of specific phenotypes can thus be performed if a particular immunologic condition is suspected. For example, T helper cells of Th1, Th2, Th17 and Treg phenotypes are known key players in the immune-inflammatory chain of molecular events leading to amplification or suppression of specific immune elements, orienting the immune response towards effective resolution or disease chronicization (17,18), according to an equilibrium in which these same cells, through the production of specific cytokines, restrict each other's own activity (19,20).

The recent personalized medicine approach, with its network view of biological systems, aims to more precisely define the individual characteristics, thus strengthening the "actionability" of diagnostic data and allowing better and more informed therapeutic choices (21-24). This approach has given momentum to the use of diagnostic homogeneous sets of biological information, highlighting the importance of using and extending diagnostic panels, where, like in the case of lymphocyte subpopulations, many pieces of a given biological function can be put together for a global view. Nevertheless, despite continuously growing knowledge about the specific role of lymphocyte cytokinergic and regulatory subpopulations, few specialized centers actually provide these analyses as panels on a routine base, a limitation possibly linked to some technical and analytical issues, especially when manipulation is needed for analyzing expression of intracellular cytokines. This issue today can be overcome by exploiting selective expression of chemokine receptors and other surface molecules on the outer lymphocyte membrane of Th1, Th2 and Th17 cells that allow a more rapid and less variable analysis. In particular, CD195 (CCR5) has been shown to be expressed by Th1 lymphocytes, while CD194 (CCR4) and CD161 are expressed by Th2 and Th17 lymphocytes respectively (25-28). However reference values for these and other regulatory subpopulations are still lacking, a further limiting factor itself for their widespread diagnostic application. The aim of this retrospective study is to contribute to the development of

reference ranges for a panel of lymphocyte subpopulations used for clinical specialist applications (Th1, Th2, Th17, Th-reg, Tc-reg, Tc-CD57⁺ and B1 lymphocytes), and known to play a role in specific conditions, such as autoimmune and degenerative diseases, including those typical of the nervous systems (10,11).

MATERIALS AND METHODS

Subjects and sample collection

Diagnostic data were retrospectively collected, pooled and analyzed from cytofluorimetric analysis of lymphocyte subpopulations of outpatient subjects referring to a community based laboratory facility in the Veneto Region in Northeast Italy, Data Medica Padova, from January 2015 to June 2016. Inclusion/exclusion criteria were applied as follows: registered cases of lymphocyte associated diseases (immunodeficiencies and lymphoproliferative disorders) were excluded from the study, as well as samples with values of total erythrocytes, total leukocytes, total lymphocytes, and major lymphocyte populations (T cells, Th, Tc and B lymphocytes) outside the normal range according to guidelines adopted by the laboratory (29,30).

The facility is part of a group of private healthcare providers operating under agreement with the public healthcare system to deliver healthcare to the public. The center is also ISO9001 certified and accredited for excellence in healthcare by Accreditation Canada International. The retrospective observational study was carried out according to good practice in clinical research guidelines and, following national Italian legislation, registered and notified to the local reference Ethics Committee (ULSS16) with the code DM 2015/01.

Typically, peripheral blood samples were collected by venipuncture in the morning hours from subjects who had been fasting from the night before. Peripheral whole blood specimens were collected in EDTA tubes from all subjects which comprised outpatient from the general community. All blood specimens were subjected to complete blood cell counts (CBC) on the day of the collection for differential count determinations on an ADVIA 2120i Hematology System analyzer with its own reference ranges and reagents were all provided by Siemens as proprietary kits for the instrument (31,32). Internal quality control checks which included runs of internal controls were performed daily, while calibration of the instrument was done at regular intervals.

Immunophenotyping of lymphocyte subsets with flow cytometry

Data from flow immunofluorimetric analysis of lymphocyte subpopulations, acquired using a four fluorescence CyFlow Cube 8 flow cytometer (Sysmex-Partec®), were collected and analyzed. Routine analytical procedures were followed for immunofluorescent labeling before flow cytometry and phenotypes were identified according to manufacturer's instructions, using the following fluorescent antibodies: T3 lymphocytes: anti-CD3 PerCP (clone UCHT1); Th lymphocytes: anti-CD3 PerCP (clone UCHT1), anti-CD4 FITC (clone MT310); Th1 lymphocytes (Th CD195⁺) (25,26): anti-CD3 APC (clone UCHT1), anti-CD4 FITC (clone MT310), anti-CD195 PE (clone R22/7); Th2 lymphocytes (Th CD194⁺) (25,26): anti-CD3 APC (clone UCHT1), anti-CD4 FITC (clone MT310), anti-CD194 PerCP-eFluor710 (clone D8SEE); Th17 lymphocytes (Th CD161⁺) (27,28,33): anti-CD3 PerCP (clone UCHT1), anti-CD4 FITC (clone MT310), anti-CD161 PE (clone DX12); Th-reg lymphocytes (34,35): anti-CD4 FITC (clone RPA-T4), anti-CD25 PE (clone BC96), anti-FOXP3 PerCP (clone PCH101); Th cells expressing CD25: anti-CD4 FITC (clone RPA-T4), anti-CD25 PE (clone BC96); Tc lymphocytes: anti-CD3 PerCP (clone UCHT1), anti-CD8 APC (clone DK25); Tc cells expressing CD25: anti-CD8 APC (clone DK25), anti-CD25 PE (clone BC96); Tc-reg lymphocytes (36): anti-CD8 APC (clone DK25), anti-CD25 PE (clone BC96), anti-FOXP3 PerCP (clone PCH101); Tc-CD57⁺ (neuroantigen specific terminally differentiated CD8⁺Tregs lymphocytes): anti-CD3 PerCP (clone UCHT1), anti-CD8 APC (clone DK25), anti CD57 FITC (clone TB01); NK cells: anti-CD3 PerCP (clone UCHT1), anti-CD8 APC (clone DK25), anti-CD56 PE (clone MOC-1); B lymphocytes: anti-CD20 PECy5 (clone B-Ly1); B1 lymphocytes: anti-CD20 PECy5 (clone B-Ly1); anti-CD27 PE (clone M-T2071), anti-CD43 FITC (clone DF-T1). Reference values, where available, were used for major populations of leukocytes (29,30,37,38). All reagents were purchased from Sysmex Partec Italia, anti-CD161 PE was from Becton Dickinson Italia SpA. Treg were immunostained using Affymetrix eBioscience human regulatory T cell staining kit (PCH101, RPA-T4-BC96) (Thermo Fischer group, Santa Clara, CA 95081), while all the other fluorescent antibodies were from DAKO Italia srl.

Statistical analysis

Analyses were performed using GraphPad Prism (v 5.01; Graph-Pad Inc., La Jolla, CA, USA) and

SigmaPlot (v 13.0; Systat Software Inc., San Jose, CA, USA). Frequency distributions were computed for each parameter and fitted with a Gaussian curve (least squares fit) to test for normality of values using a D'Agostino&Pearson omnibus K2 test (null hypothesis: non-Gaussian distribution). If parameters followed a Gaussian distribution, means, standard deviation and 95% confidence intervals (reference range) were calculated. If the result of D'Agostino&Pearson test were significant, the data were considered to have a non-Gaussian distribution, but given that the data have a sample size greater than 50 and their distribution is similar to a normal, or a Poisson, or a gamma distribution (according to the central limit theorem and the law of large numbers (39,40)), then confidence intervals were calculated with an alpha of 0.05 that is, with a probability of 95%. Statistical comparisons for lymphocyte subsets for gender and age groups were computed using one-way analysis of variance (ANOVA) followed by *post-hoc* tests for selected comparisons or two-tailed Student's *t* test. Significance level was set at $p < 0.05$. Additional details are provided in the figure captions, where appropriate.

RESULTS

Sample description

Peripheral blood leucocytes data from an outpatient population in Northeast Italy were collected for a retrospective observational study of lymphocyte subsets identified with flow immunocytometry. Data were collected in a timeframe from January 2015 to June 2016 and inclusion/exclusion criteria were applied as described in the methods section. The initial sample consisted of 365 Caucasian adults (age mean 41.3 years; age range: 19~58 years), 111 males and 254 females. Samples with out-of-range cell counts of erythrocytes, leukocytes and major lymphocyte populations were excluded from the analysis. Following the application of exclusion criteria the sample was reduced to a total of 78 (age mean 42 years), 24 males and 54 females (see Table I).

Immune cell counts and major lymphocyte subpopulations

Mean values of white blood cells (WBC), neutrophils, lymphocytes, monocytes, eosinophils and basophils, both absolute numbers and percentage of WBC, were obtained from the hemochrome (CBC) of the subjects and were used to calculate the absolute values of lymphocyte subpopulations from the percentages obtained with cytofluorimetric analysis (see Table II). The graphical

Table I. Description of the subjects used for the analysis before and after application of exclusion criteria

	N total	N male	N female	Age (mean±SD)
Initial sample before application of exclusion criteria	365	111	254	41.3±9.1
After exclusion of samples outside normal range of Red blood cells (4200~5400 cells/μl)	298	101	197	41.9±8.8
After exclusion of samples outside normal range of White blood cells (4800~10800 cells/μl)	189	73	116	41.7±8.9
After exclusion of samples outside normal range of Total Lymphocytes (1500~4000 cells/μl)	104	36	68	41.9±8.1
After exclusion of samples outside normal range of Total T Lymphocytes (1000~3200 cells/μl)	100	34	66	42.0±8.7
After exclusion of samples outside normal range of Total Th Lymphocytes (500~2200 cells/μl)	98	33	65	41.9±8.2
After exclusion of samples outside normal range of Total Tc Lymphocytes (300~1520 cells/μl)	84	28	56	41.7±8.4
After exclusion of samples outside normal range of Total B Lymphocytes (105~560 cells/μl)	78	24	54	42±8.5
Sample following inclusion/exclusion criteria	78	24	54	42±8.5

Normal ranges are as previously defined (29,30).

Table II. Major peripheral blood leukocytes and lymphocyte subpopulations from CBC and cytofluorimetric analysis, respectively, in the selected sample of 78 adults

	N	Median	Mean	SD	Lower 95% CI of mean	Upper 95% CI of mean
WBC/μl	78	6745.0	6925.9	1255.5	6643.0	7209.0
% NEU	78	55.1	55.2	8.2	53.3	57.0
NEU/μl	78	3690.0	3854.7	1076.4	3612.0	4097.0
% LYMPH	78	33.8	33.6	7.1	32.0	35.2
LYMPH/μl	78	2205.0	2298.8	550.9	2175.0	2423.0
% MONO	78	5.9	6.0	1.6	5.7	6.4
MONO/μl	78	395.0	411.0	113.9	385.3	436.7
% EOS	78	2.3	2.7	1.9	2.2	3.1
EOS/μl	78	155.0	177.3	117.1	150.9	203.7
% BASO	78	0.5	0.6	0.2	0.5	0.6
BASO/μl	78	35.0	37.6	17.1	33.7	41.4
% T3	78	70.5	70.3	8.5	68.4	72.3
T3/μl	78	1607.0	1604.8	375.0	1520.0	1689.0
% Th	78	44.0	43.8	7.5	42.1	45.5
Th/μl	78	1005.0	997.0	258.1	938.8	1055.0
% Th-CD25+	78	1.0	1.1	0.8	0.9	1.3
Th-CD25+/μl	78	20.5	24.2	18.3	20.1	28.3
% Tc	78	22.0	23.4	7.1	21.8	25.0
Tc/μl	78	474.5	536.2	214.2	487.9	584.5
% Tc-CD25+	78	0.6	0.6	0.3	0.6	0.7
Tc-CD25+/μl	78	13.0	14.0	8.3	12.2	15.9
% B	78	9.0	10.5	3.8	9.6	11.3
B/μl	78	207.0	241.3	111.5	216.7	266.7
% NK	78	15.0	16.9	8.3	15.0	18.8
NK/μl	78	373.5	373.3	198.3	343.0	452.4

Values are mean, median, standard deviation and 95% confidence interval. Values are expressed as circulating cells per μl of peripheral venous blood and as percentage of total white blood cells for leukocyte, or total lymphocytes for lymphocyte subpopulations, respectively. WBC, White Blood Cells; NEU, neutrophils; LYMPH, lymphocytes; MONO, monocytes; EOS, eosinophils; BASO, basophils.

distribution of values from the hemochrome and the cytofluorimetric analysis of standard lymphocyte subpopulations: T3, Th, Tc, B, NK, (see Fig. 1A and 1B, respectively) is represented using the nonparametric distribution of sigma plot box, showing median with 25th and 75th percentile borders, error bars represent 10th and 90th percentile and outlier distribution with single dots. Values of IL-2 receptor expressing, activated T lymphocytes (Th-CD25⁺, Tc-CD25⁺) were also considered in this analysis, to evaluate the level of immune activation of the samples. Collectively, the values of standard immune cells indicate a high level of uniformity of the samples. Together with the low degree of IL-2 receptor expression (CD25⁺), this suggests that the subjects were likely not to have ongoing major immune alterations or dysfunctions. There were no significant differences between sexes and between the two age ranges considered

(above or below 40yrs) (see Fig. 1A and B).

Cytofluorimetric immunophenotyping of cytokinergic and regulatory lymphocyte subpopulations

The lymphocyte subpopulations object of the study were analyzed with the immunocytofluorimetric procedures described in the methods. Mean values, standard deviation, standard error and confidence interval of percentage and absolute counts of the different lymphocyte subsets were calculated and nonparametric, as well as Gaussian distributions of data points analyzed. Although non parametric analysis is the normal choice for studying the distribution of data points from data affected by large numbers of variables, as in the case of circulating blood cells, a Gaussian analysis was also performed for each subset of cells to distribute and characterize lymphocyte subpopulations according to their level of intrinsic variability/instability. In particular Gaussian distribution of data was assessed (see Table IIIA, B and Fig. 2). Values for %Th1, Th1/ μ L, %Th2, Th2/ μ L, %Th17, Th17/ μ L, %Th-reg, Th-reg/ μ L display a Gaussian distribution, while the others do not, suggesting that the latter might be more sensitive to subtle stimuli or display a more dynamic behavior by nature. The confidence intervals were calculated with an alpha of 0.05 that is, with a probability of 95%. The distribution of lymphocyte subpopulations between sexes and between the two age ranges considered (above or below 40 yrs) did not show any significant statistical difference (Fig. 3A and 3B).

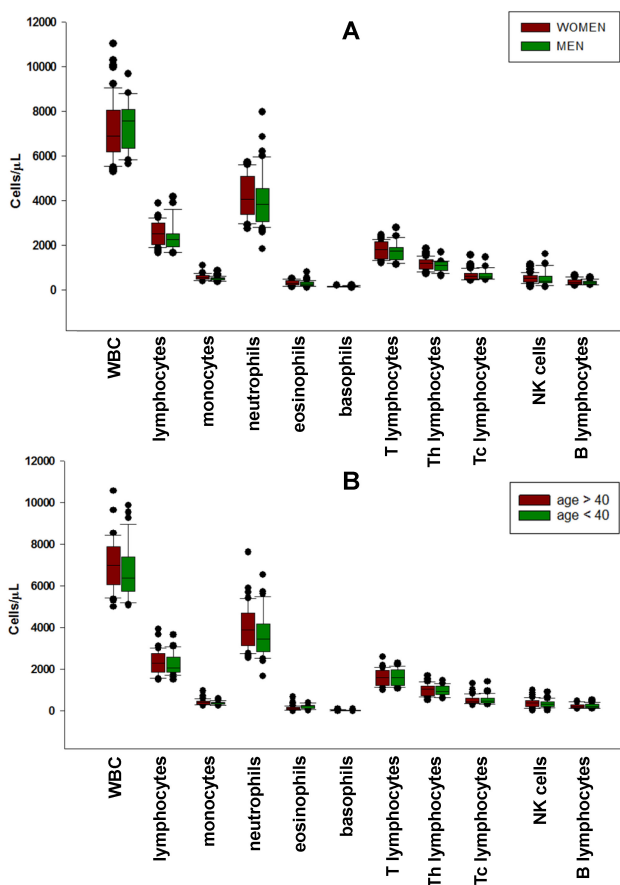


Figure 1. Distribution of major peripheral blood leukocytes and lymphocyte subpopulations between sexes (A) and the two age ranges considered (above or below 40 yrs) (B). Box plots represent median with 25th and 75th percentile borders, error bars represent 10th and 90th percentile and outlier distribution with single dots. No significant differences between sexes and age were found.

DISCUSSION

This retrospective study was designed to establish reference range values of a panel of lymphocyte subpopulations known for their functional and regulatory role in many facets of immune dysregulations, but that have not yet been consistently included in routine assays for immune function characterization. Variations in population and environmental factors need to be addressed individually by region, as these may play a major role in defining the so-called normal reference ranges and overall immune status (37,38). The present study focused on determining reference ranges for lymphocyte subsets in a cohort of outpatients representative of male and female adult Caucasians in Northeast Italy. In particular, we verified our established reference ranges, widely used for the routine lymphocyte panel (29,30,37,38) and extended the analysis to additional lymphocyte subpopulations which, although regularly performed in our laboratory,

Table IIIA. Mean, standard deviation, minimum/maximum and 95% confidence interval with D'Agostino&Pearson test of peripheral blood lymphocytes subsets from cytofluorimetric analysis in the selected 78 sample

Parameters	%Th1	Th1	%Th2	Th2	%Th17	Th17	%Th-reg	Th-reg	Th1/Th2	Th-reg/Th17
D'Agostino&Pearson omnibus normality test										
p value	0.093	0.156	0.067	0.121	0.295	0.073	0.127	0.076	-	-
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	-	-
Mean	7.3	157.8	5.4	118.2	10.5	221.6	0.7	15.1	1.4	0.1
SD	3.0	60.3	2.5	52.2	4.4	90.2	0.4	10.2	0.5	0.1
Minimum	2.3	55.0	1.5	32.0	1.3	32.0	0.0	0.0	0.5	0.0
Maximum	15.8	317.0	12.2	261.0	21.6	489.0	1.9	45.0	2.7	0.3
Confidence Interval (alpha=0.05)	0.67	13.60	0.56	11.75	0.98	40.70	0.10	2.31	0.11	0.02
Reference range	6.66~8.01	144.20~171.40	4.86~5.98	106.40~129.90	9.50~11.47	201.30~242.00	0.59~0.79	12.79~17.41	1.33~1.54	0.06~0.11

Values are expressed as circulating cells per µl of peripheral blood and as percentage of lymphocytes for lymphocyte subpopulations. In the upper part the results of the D'Agostino & Pearson omnibus normality test for the Gaussian distribution are shown. p> 0.05 stands for a Gaussian or normal distribution.

Table IIIB. Mean, standard deviation, minimum/maximum and 95% confidence interval with D'Agostino&Pearson test of peripheral blood lymphocytes subsets from cytofluorimetric analysis in the selected 78 sample

Parameters	%Th CD25+	Th CD25+	%Tc CD25+	Tc CD25+	%Tc-reg	Tc-reg	%Tc CD57+	Tc CD57+	%B1	B1
D'Agostino&Pearson omnibus normality test										
p value	<0.0001	<0.0001	0.032	0.0004	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Passed normality test (alpha=0.05)?	No	No	No	No	No	No	No	No	No	No
Mean	1.1	24.2	0.6	14.0	0.3	5.8	4.6	103.7	1.5	33.7
SD	0.7	18.3	0.3	8.2	0.2	4.6	4.7	114.1	0.8	22.8
Minimum	0.2	5.0	0.1	3.0	0.0	0.6	0.3	7.0	0.5	8.0
Maximum	5.0	118.0	1.8	43.0	1.2	23.0	30.0	757.0	5.0	98.0
Confidence Interval (alpha=0.05)	0.17	4.06	0.07	1.83	0.04	1.03	1.05	25.32	0.19	5.07
Reference range	0.92~1.25	20.13~28.26	0.57~0.72	12.21~15.87	0.23~0.32	4.73~6.80	3.53~5.65	78.40~129.03	1.27~1.66	28.62~38.76

Values are expressed as circulating cells per µl of peripheral blood and as percentage of lymphocytes for lymphocyte subpopulations. In the upper part the results of the D'Agostino & Pearson omnibus normality test for the Gaussian distribution are shown. p>0.05 stands for a Gaussian or normal distribution.

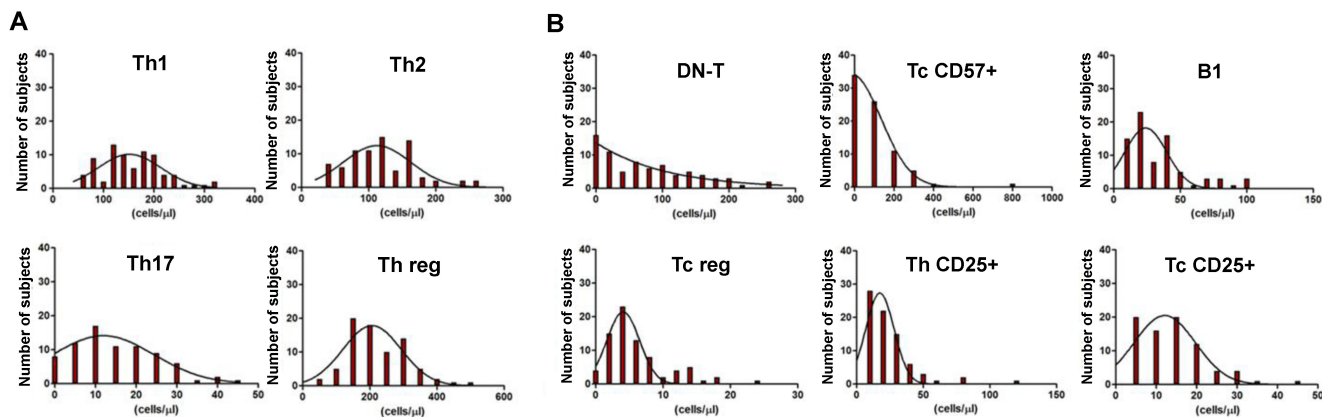


Figure 2. Distribution of lymphocyte subpopulations for Gaussian analysis. Gaussian distribution of lymphocyte subpopulations: Th1, Th2, Th17 and Th-reg (A). Non-Gaussian distribution of lymphocytes subpopulations: Tc-CD57⁺, B1 cells, Tc-reg, Th-CD25⁺ and Tc-CD25⁺ (B).

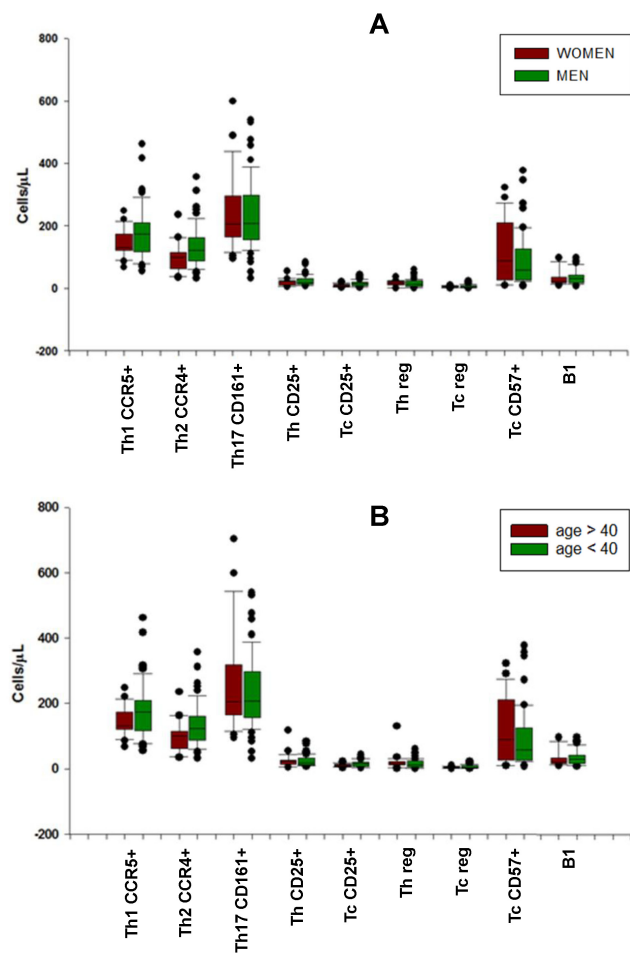


Figure 3. Distribution of lymphocyte subpopulations between sexes (A) and the two age ranges considered (above or below 40 yrs) (B). Box plots represent median with 25th and 75th percentile borders. Error bars represent 10th and 90th percentile and outlier distribution with single dots. No differences between sexes and age were found.

still lack precise reference values.

Stringent exclusion/inclusion criteria were applied, in order to limit as much as possible pathological abnormalities due to major immune and blood dysfunctions. Application of these criteria reduced the sample to 21% of the initial population. Analysis of extended panels of lymphocytes is usually requested as a second line diagnostic for suspected dysfunctions. It is thus not surprising that most patients, although not ill enough to be hospitalized, suffered from alterations in blood cell counts. Although the numerosity was reduced to 78, this value was sufficient to ensure statistical power for analysis and, despite the large reduction, the characteristics of the population were not significantly affected, both in terms of mean age and gender.

Mean values of “standard” leukocytes and lymphocytes from the selected population were in accordance with published values (29-32,37,38), regardless of age and sex: total leukocytes, neutrophils, eosinophils, basophils, total lymphocytes, T lymphocytes, Th, Tc, B lymphocytes and Natural Killer cells (Table II and Fig. 2), thus validating the reference values used in the laboratory.

Besides the main standard lymphocyte subpopulations, the major group of phenotypes analyzed was characterized by immune regulatory functions exerted either by specific cytokine-producing lymphocytes (Th1, Th2 and Th17) (10,11), by lymphocytes with direct suppressive functions (Th and Tc regulatory cells, Tc-CD57⁺) (34-36,41-44), or by lymphocytes with mixed mechanisms (B1 lymphocytes) (45-46). All these sub-classes are believed to play an important role in chronic immune dysregulations and are included in diagnostic cytofluorimetric lymphocyte panels used for such conditions (47,48), including neuro-immune

dysregulations (49-51).

Cytokine producing T helper lymphocytes are involved in the early steps of the immune cascade. By activating specific pathways and inhibiting others, these lymphocytes are key players in the immune response and variations in their subsets can be suggestive of specific pathological conditions (52). Based on cytokine profiles produced upon activation, T helper lymphocytes can be sub-classified into Th1, Th2, Th17 and a growing number of other minor sets, known to characterize immune responses in several diseases (10,20,53-56). Immune fluorescent staining of specific surface molecules was used to identify Th subsets (25-28). Th1 mean value, as expected, was higher than Th2 mean value, while the value of Th17 lymphocytes was consistently higher than the other two cytokinergic lymphocyte subgroups. Accordingly, all ratios normally used to evaluate Th cytokinergic and regulatory patterns (Th1/Th2, Th-reg/Th17) are within published ranges (57-61). Nevertheless the use of a simplified test for lymphocyte subpopulations, where phenotypes are based upon surface antigen expression rather than actual intracellular cytokine expression, should always be acknowledged in order to account for potential risks associated with this assumption.

Th-reg and Tc-reg lymphocytes are implicated in negative feedback to immune activation and are suggested to play a central role in the immune modulation associated with specific pathologic conditions (34-36). In this study their values are the lowest, which is not surprising, given their fine tuning role in immune functions.

Tc-CD57⁺ (also known as T-reg neuroantigen specific) lymphocytes have been identified as cytotoxic effector memory T cells capable of immediate functional activity, with high susceptibility to activation-induced cell death and immunosuppressive capacity (43,44). Increased numbers of Tc-CD57⁺ cells are associated with a wide range of chronic diseases and some viral infections (44), while their neurotropic nature also suggests a role in neuro-immune dysregulations (62,63). Their values are rather variable in this study, perhaps related to the unpredictability of an individuals' immune history.

B1 cells are the final regulatory lymphocyte subpopulation included in the panel. These lymphocytes are identified as CD20⁺CD27⁺CD43⁺ B cells and have been suggested to constitute a distinct B cell lineage with a unique set of characteristics that includes constitutive production of auto-reactive antibodies, regulatory cytokines like IL-10 and a strong ability to activate other regulatory lymphocytes (47,48,64).

Taken together these results contribute to establishing reference ranges for each type of lymphocyte subpopulation examined. In particular, the results indicate that values from most subpopulations (Th1, Th2, Th17, Th-reg) display a Gaussian distribution, which characterizes a homogeneous population. Values for the remaining subpopulations (Tc-reg, Tc-CD57⁺, B1), although not displaying a Gaussian distribution, can still be statistically considered as normally distributed. Such distribution allows for their use as reference range and favors their diagnostic application. Further, statistical significance of the distributions, despite the relatively low number of subjects used in the study, considered together with values of standard deviation, supports the view that most lymphocyte subpopulations analyzed, in normal conditions represent rather stable proportions of lymphocytes. Tc-CD57⁺ cells represent a predictable exception, given their intrinsic variability (43,44,62,63). The mean, median and standard deviation values, as well as reference ranges, or confidence intervals did not differ significantly between sexes or the two age groups considered (19~40 and 41~58), suggesting their applicability for adults of either sex.

Immune cells are functionally related and changes in a regulatory cell type often lead to a network phenomenon, their numbers should thus be considered as a whole rather than single biomarkers. Positive and negative relationships between lymphocytes should thus be taken into account when presenting the results of a diagnostic lymphocyte panel. Some of the changes might also be different from what is expected from known relationships and these might be associated to specific pathologic conditions.

The standardization process thus includes not only the definition of reference ranges, but also a clear definition of the known functional relationships among lymphocytes, especially if paneling regulatory and cytokinergic cells (see Fig. 4), as a guideline to follow for the interpretation of results. Progressing from the idea of diagnostic biomarkers to a vision of biomarker networks enhances the "actionability" of diagnostic data (24,65) and is a key passage in the application of a systems biology view for the interpretation of collective diagnostic data, an approach strategically functional in the path to personalized medicine (21-23,66-68).

Further studies are required with larger samples to consolidate the reference range values identified and a constant update of the immune cell network will be necessary for the correct use of the panel. Validation of these values in other populations will also be needed and

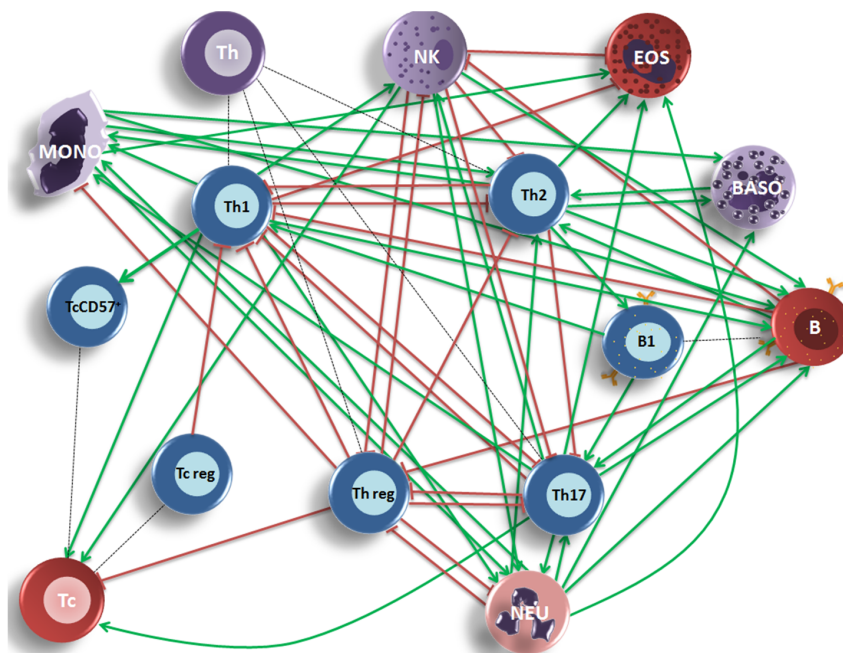


Figure 4. A systems medicine view of the regulatory lymphocyte subpopulations panel. Activatory (A, green arrows) and Inhibitory (I, red lines) relationships between circulating cells were identified according to cellular or cytokinergic interactions previously described. In some cases different states of the same cell type produce conflicting stimuli and the relative relationship was thus not assigned in the figure. Th1 lymphocytes - A: Tc, Tc-CD57⁺, NK, MONO, NEUT; I: Th2, Th17, EOS (69-82). Th2 lymphocytes - A: B lymphocytes, B1 lymphocytes, EOS, BASO, MONO; I: Th1, Th17 (77-79,83-88). Th17 lymphocytes - A: Tc, B lymphocytes, NK, MONO, NEUT, EOS; I: Th1, Th-reg (73,89-96,109). Th-reg lymphocytes - I: Th1, Th2, Th17, Tc, NK, MONO, NEUT (56,83,88,92,97-102). B lymphocytes - A: Th2, Th17; I: Th1, Th-reg, NK (103-106). B1 lymphocytes - A: Th1, Th17 (107,108). Tc-reg lymphocytes - I: Th1(42). NK cells - A: Tc, B cells, NEUT; I: Th2, Th-reg, EOS (76,78,86,89,93,95,110). MONO - A: Th2, B cells, BASO, NEUT, EOS (77,106,111,112). NEUT - A: Th2, Th17, B cells, MONO, EOS, BASO; I:Th-reg (77,78, 93,94,113). Artwork was created with Microsoft Office PowerPoint 2007, standard blood cell cartoon symbolic representation was used.

is part of our future research plan. The present reference range values can in fact only be applied to the selected age group (adults). Specific studies are required to verify them across age groups (i.e. children, the elderly), as well as in non-Caucasian populations.

ACKNOWLEDGEMENTS

The authors thank Dr Sebastian Desando for kindly assisting with the revision of the manuscript, and Dr Ezio Tirloni from Sysmex-Partec for assistance in preparing figures illustrating immune-cytofluorimetric plots.

CONFLICTS OF INTEREST

The authors don't have financial conflict of interest.

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