

Determination of Reference Values for Double-Negative T Lymphocytes in Cuban Adults

Carlos A. Villegas-Valverde MD MS, Elena Kokuina MD PhD, Martha C. Breff-Fonseca MD

ABSTRACT

INTRODUCTION Double-negative T lymphocytes act as immunomodulators in immune response. This subpopulation is rare in blood but important in the immunopathogenesis of autoimmune diseases, viral infections, cancer and transplant rejection. These disorders have been studied in Cuba using flow cytometry, but normal values of these cells have not yet been established.

OBJECTIVE Estimate preliminary reference values for double-negative T lymphocytes according to sex and age in Cuban adults.

METHODS A cross-sectional study was carried out in a population of 182 healthy adult residents of Havana: 93 women and 89 men aged 18–80 years with no chronic diseases, toxic habits (smoking, excessive alcohol or caffeine intake) or medications that might alter quantity or functioning of immune-system cells. Peripheral blood was drawn to determine immunophenotype with monoclonal antibodies. The phenotype of double-negative T lymphocytes was quantified as CD45⁺/CD3⁺/CD4⁻/CD8⁻/CD56⁻ using a Gallios flow cytometer (Beckman-Coulter, France). Medians and ranges (to the 5th and 95th percentiles) were calculated for sex and age, for both percentages and absolute values. To evaluate the effects of sex and age, both variables as well as their interaction were included in a linear model.

RESULTS Respective median and range values were total percentage values 3.4 (1.6–7.4) and total absolute values (cells/ μ L) 57.5 (23.0–157.0). The effect of age on lymphocyte values (percentage and absolute) was significant, with lower numbers in the 51–80 years' age group ($p < 0.001$). Percentage values according to age group were: 18–25 years, 3.8 (2.2–7.4); 26–50 years, 3.7 (1.7–8.7); and 51–80 years 2.6 (1.3–6.6). Absolute values by age group were: 18–25 years, 90 (32.6–163.7); 26–50 years, 65 (28.8–184.0); and 51–80 years 38.5 (17.9–90.1). Desegregating data by sex and age: percentage of women aged 18–25 years 5.2 (2.1–7.8), 26–50 years 4.0 (1.8–7.7), and 51–80 years 2.5 (1.3–5.8); percentage of men aged 18–25 years 3.4 (2.3–7.3), 26–50 years 3.8 (1.5–8.7), and 51–80 years 2.6 (1.2–7.3). Absolute values: women aged 18–25 years 112.0 (40.0–153.1), 26–50 years 67.0 (26.7–138.3), and 51–80 years 40 (18.6–92.0); and men aged 18–25 years 71.5 (32.1–166.7), 26–50 years 61.5 (29.9–188.7), and 51–80 years 36 (13.5–81.7). The low sex*age interaction confirms these differences occur in both men and women. Values decrease with age, with a more abrupt fall starting at 50 years.

CONCLUSIONS Estimated reference values were determined for absolute values and relative proportions of double-negative T lymphocytes in healthy Cuban adults according to sex and age. Age was found to have a significant effect.

KEYWORDS Reference values, T lymphocytes, flow cytometry, immunology, Cuba

INTRODUCTION

Flow cytometry has enabled study of lymphocyte populations and their heterogeneity. In lymphocyte phenotyping, two types of classical T-lymphocyte populations (TCD4⁺ and TCD8⁺) were initially reported. These were considered as two unique, mutually exclusive T-cell subpopulations, since early cytometry allowed for only two or three simultaneous markings on a single cell.[1] The TCD3⁺ lymphocytes that do not express markers CD4 and CD8 are known as “double-negative” T cells (DNT cells). They were recently discovered and are characterized by a mature immunophenotype (CD45⁺/CD3⁺/CD4⁻/CD8⁻/CD56⁻) and express clonotypic T-cell receptors (CTR) of type $\alpha\beta$ or type $\gamma\delta$. These cells are found in greater proportion in the blood and lymph nodes, although they reportedly account for 3%–7% of total lymphocytes in peripheral blood.[2–5]

The physiological functions of DNT lymphocytes are not yet fully understood. The immunomodulation attributed to them is based on their ability to suppress the functions of simple positive T cells and their cytotoxicity for tumor cells and cells infected

by viruses.[2,3] In some immune-system diseases, variations were found in total numbers of DNT lymphocytes or their functions were altered. Although only small quantities of these lymphocytes are found in blood, they play an important role in various diseases' immunopathogenesis. Their blood levels provide criteria for diagnosing autoimmune lymphoproliferative syndrome and can provide a prognostic biomarker for cancer or effective therapeutic targets for these diseases.[2–7]

In order to make clinical decisions about autoimmune diseases, viral infections, solid tumors and hematopoietic diseases, transplant rejection, alterations of lymphocytic homeostasis and immunodeficiencies,[3–8] absolute and relative levels of lymphocytes are quantified through flow cytometry, so normal DNT-lymphocyte counts must be determined. This subpopulation can be identified without additional reagent costs because the values can be quantified through conventional analysis of T, B and natural killer (NK) cells.

The purpose of this study was to determine the normal range of values of DNT lymphocytes in a population of healthy Cuban adults and the relation of these values with age and sex—such information could inform diagnosis and prognosis of multiple diseases.

METHODS

Design, subjects and sample selection A cross-sectional study was conducted in 2017–2018 at the Hermanos Ameijeiras Clinical-Surgical Teaching Hospital and The National Oncology &

IMPORTANCE Normal values of double-negative T lymphocytes in Cuban adults are useful for studying immune-system diseases, cancer and transplant rejection and for avoiding subregistration when using flow cytometry to analyze T, B and NK cells.

Radiobiology Institute, Havana, Cuba. Subjects included 182 adult residents of Havana, aged 18–80 years (median age 40), 93 women and 89 men, all reportedly healthy, who were accompanying patients at hospital immunology services and who agreed to participate via written informed consent. Persons were excluded who smoked, consumed daily >40 g of alcohol or its equivalent weekly; drank more than 4 cups of coffee daily; were pregnant; had recent or recurring infections in the previous 6 months or autoimmune or neoplastic diseases; or had taken antibiotics, immunostimulators, immunosuppressants or anti-inflammatory drugs in the past 6 months.

Flow cytometry Four mL of peripheral blood was extracted by antecubital venipuncture and deposited in cytometry tubes with the anticoagulant ethylenediaminetetraacetic acid (EDTA). Sample preparation adhered to the manufacturer's specifications for cell-surface immunophenotyping, using a protocol for no-wash red blood cell lysis with a Versalyse buffer (Beckman-Coulter, France).

An eight-color Beckman-Coulter Gallios flow cytometer (Beckman-Coulter, France) was used. The following fluoro-chrome-conjugated monoclonal antibodies were used: anti-CD45-AA750/CD3-FITC/CD4-PC5.5/CD8-AA700/CD56-PE (all Beckman-Coulter, France). The DNT lymphocyte population was defined by the CD45⁺/CD3⁺/CD4⁻/CD8⁻/CD56⁻ immunophenotype. The data obtained were processed with Kaluza Analysis V1.5a software, with a minimum of 50,000 acquisition events. Absolute values were determined through a double platform and application of the following equation:

$$\text{absolute value (cells/}\mu\text{L)} = (\text{lymphocytes/}\mu\text{L of hemogram}) \times (\% \text{ DNT}) \div 100.$$

Statistical analysis Variables calculated include descriptive statistics, absolute and relative frequencies, mean, median and percentiles. To define value range, the normal distribution of variables was evaluated by the Shapiro-Wilk test. Most variables did not follow a Gaussian distribution. The effects of sex, age and their interaction were evaluated using a linear model. Reference values were expressed through the median and the 5th and 95th percentiles and stratified by sex and age group (18–25, 26–50, and 51–80 years).

Ethical considerations The project was approved by the research ethics committee of the Hermanos Ameijeiras Clinical-Surgical Teaching Hospital. Written informed consent was obtained from study participants after they received an explanation of the study's possible risks and benefits. The information was protected under principles of confidentiality without revealing participant identity. Diagnostic methods were selected based on the principle of maximum benefit, international and national standards, and accessibility of materials.

RESULTS

Percentages and absolute values of DNT lymphocytes are presented in Table 1. They were significantly lower in the 51–80 age group.

In both percentage and absolute values, the effect of age was highly significant, as evident in the variations observed between the oldest age group (51–80) and the two younger age groups (18–25 and 26–50).

DISCUSSION

At the time of this writing, there were no studies on reference-value ranges for DNT lymphocytes in the Cuban population. Two publications reported that quantification of these cells is needed to diagnose autoimmune lymphoproliferative syndrome.[6,7] Although it is common for each laboratory to define specific ranges, there should be steady progress toward this goal. Given the dearth of data on reference values, and the urgency to collect such data, this study aims to propose ranges that amount to preliminary approximations for application at the national level.

Multiple factors are known to intervene in the variability of physiological ranges for these lymphocytes and for all immune-system cells. The most studied factors are age and sex.[5] In this study, the effects of both age and sex were evaluated, and it was confirmed that the effect of age was significant, especially differences between those 51–80 years compared to those ≤50. The low sex/age interaction confirms that these differences occur in both men and women and may be due to immunosenescence and thymic involution, the consequences of which become more evident near the sixth decade of life. In a study of healthy Cubans, a negative correlation was found between age and global T-cell count.[9]

The ranges obtained were wider and had higher medians than those reported internationally. These differences could be due to effects of lifestyle, diet and environmental conditions of the populations studied,[5,10] or because, unlike ours, the other studies did not exclude individuals with unhealthy habits or those consuming medications that could have affected immunological variables.[9] More local studies are needed to determine reference values of cells that can be used provisionally until studies with broader population representation can be conducted.

Table 1: Normal ranges (5th and 95th percentiles) for double-negative T lymphocytes by sex and age group (n = 182)

Age groups in years / Sex	Men (n = 89)		Women (n = 93)		Total	
	Median	Percentiles (5.0–95.0)	Median	Percentiles (5.0–95.0)	Median	Percentiles (5.0–95.0)
Range in percentage values (relative to total CD3⁺CD56⁻ lymphocytes)						
18–25 (n = 30)	3.4	2.3–7.3	5.2	2.1–7.8	3.8	2.2–7.4
26–50 (n = 94)	3.8	1.5–8.7	4.0	1.8–7.7	3.7	1.7–8.7
51–80 (n = 58)	2.6	1.2–7.3	2.5	1.3–5.8	2.6	1.3–6.6
Total	3.5	1.5–8.1	3.3	1.6–7.2	3.4	1.6–7.4
Range in absolute values (relative to total lymphocytes, cells/μL)						
18–25 (n = 30)	71.5	32.1–166.7	112.0	40.0–153.1	90.0	32.6–163.7
26–50 (n = 94)	61.5	29.9–188.7	67.0	26.7–138.3	65.0	28.8–184.0
51–80 (n = 58)	36.0	13.5–81.7	40.0	18.6–92.0	38.5	17.9–90.1
Total	59.0	23.0–185.0	57.0	22.7–132.2	57.5	23.0–157.0
Effects (DNT%) ^a	F(sex) = 1.47 (p = 0.23); F(age) = 7.27 (p <0.01); F(sex*age) = 1.64 (p = 0.20)					
Effects (DN#) ^b	F(sex) = 0.32 (p = 0.57); F(age) = 28.74 (p <0.001); F(sex*age) = 0.19 (p = 0.67)					


^a Percentage values (relative to total CD3⁺CD56⁻ lymphocytes)

^b Absolute values (relative to total lymphocytes, cells/μL)

F: Fisher's test for linear models

Constraints in our study included the fact that the sampling was based entirely on residents of Havana with no one from other provinces; also, additional serological studies were not conducted, which reduced the possibility of diagnosing some chronic and infectious diseases that could have affected DNT lymphocyte values.

CONCLUSIONS

References were estimated for absolute values and proportions of double-negative T lymphocytes in healthy Cuban adults according to sex and age, and the importance of the effect of age was established. Absolute and relative concentrations of DNT lymphocytes were highest in younger age groups. 

REFERENCIAS

1. Melzer S, Zachariae S, Bocsi J, Engel C, Löffler M, Tárnok A. Reference intervals for leukocyte subsets in adults: results from a population-based study using 10-color flow cytometry. *Cytometry B Clin Cytom*. 2015 Jul-Aug;88(4):270–81.
2. De Tullio G, Strippoli S, Angarano R, De Fazio V, Sgherza N, Negri A, et al. $\alpha\beta$ -Double negative CD4/CD8 (CD56) T cell (DNTs) in metastatic melanoma: basal frequency and behaviour during Ipilimumab treatment. Preliminary evaluations. *J Transl Med* [Internet]. 2015 [cited 2020 Jan 21];13(Suppl 1):O10. Available at: <https://doi.org/10.1186/1479-5876-13-s1-o10>
3. Haug T, Aigner M, Bruns H, Peuser M, Gebhardt D, Berger J, et al. Human double-negative regulatory T cells modulate effector function of conventional T cells by selectively blocking mTOR signaling. *Blood* [Internet]. 2018 Nov 29 [cited 2020 Jan 21];132(Suppl 1):2410. Available at: <https://doi.org/10.1182/blood-2018-99-112943>
4. Voelkl S, Gary R, Mackensen A. Characterization of the immunoregulatory function of human TCR- $\alpha\beta$ CD4CD8 double-negative T cells. *Eur J Immunol* [Internet]. 2011 [cited 2020 Jan 21];41:739–48. Available at: <https://doi.org/10.1002/eji.201040982>
5. Apoil PA, Puissant-Lubrano B, Congy-Jolivet N, Peres M, Tkaczuk J, Roubinet F, et al. Reference values for T, B and NK human lymphocyte subpopulations in adults. *Data in Brief* [Internet]. 2017 [cited 2020 Feb 15];12:400–4. Available at: <https://doi.org/10.1016/j.dib.2017.04.019>
6. Casado Hernández I, Marsán Suárez V, Díaz Domínguez G, Macías Abraham C. Utilidad diagnóstica de la evaluación de linfocitos T CD4+ CD8+ TCR $\alpha\beta$ + en el Síndrome Linfoproliferativo Autoinmune. *Rev Cubana Hematol Inmunol Hemoter*. 2017;33(2):1–4. Spanish.
7. Casado Hernández I, Marsán Suárez V, Díaz Domínguez G, Macías Abraham C, Machín García S. Diagnóstico por citometría de flujo del Síndrome Linfoproliferativo Autoinmune. *Rev Cubana Hematol Inmunol Hemoter* 2017;36(Suppl):1–8. Spanish.
8. Wang Y, Lu W, Li A, Sun Z, Wang L. Elevated CD3 low double negative T lymphocyte is associated with pneumonia and its severity in pediatric patients. *Peer J* [Internet]. 2018 Dec 18 [cited 2020 Feb 15];6:e6114. Available at: <https://doi.org/10.7717/peerj.6114>
9. García Verdecia B, Saavedra Hernández D, Lorenzo-Luaces P, de Jesús Badía Álvarez T, Leonard Rupalé I, Mazorra Herrera Z, et al. Immunosenescence and gender: a study in healthy Cubans. *Immun Ageing* [Internet]. 2013 Apr 30 [cited 2020 Feb 15];10(1):16. Available at: <https://doi.org/10.1186/1742-4933-10-16>
10. García-Dabrio C, Pujol-Moix N, Martínez-Pérez A, Ubeda J, Martínez C, Buil A, et al. Valores de referencia de las subpoblaciones linfocitarias en la población sana española. *Hematológica*. 2009;94(4):40. Spanish.

THE AUTHORS

Carlos Agustín Villegas-Valverde, physician with dual specialties in family medicine and immunology, and master's degrees in infectious diseases and medical education. Associate researcher, Immunology Laboratory of the National Institute of Oncology and Radiobiology, Havana, Cuba. <https://orcid.org/0000-0001-7478-0482>

Elena Kokuina, physician specializing in immunology, with a doctorate in medical sciences, clinical laboratory department, Hermanos Ameijeiras Clinical-Surgical Teaching Hospital, Havana, Cuba. Full professor, Medical University of Havana, Cuba. <https://orcid.org/0000-0002-3651-7445>

Martha Camila Breff-Fonseca, physician and clinical laboratory specialist, Hermanos Ameijeiras Clinical-Surgical Teaching Hospital, Havana, Cuba.

Submitted: May 8, 2020

Approved for publication: September 16, 2020

Disclosures: None