Introduction

Classically, T cells have been classified according to the cell surface markers CD4 and CD8. The expression of these proteins is considered to be a mutually exclusive event reflecting the specific functions of each major T cell population in peripheral blood: CD4+ or helper T cells and CD8+ or cytotoxic T cells. However, with the use of multiparametric cellular analysis methods, a variety of minor T cell subpopulations have been described [1], such as mature CD4+CD8+ or double-positive T cells (DPTs) [2,3]. This T cell phenotype was initially described in the thymus, where more than 80% of thymocytes expressed both CD4+CD8+, which later commit to one cell lineage (CD8+ or CD4+) after interaction with human leukocyte antigen (HLA) class I or II molecules,
respectively [4]. The origin of DPTs in the peripheral blood of healthy individuals has been attributed to the premature release of CD4+CD8+ T cells from the thymus to the periphery [5–7]. However, additional studies have suggested that DPTs could originate from the acquisition of the second marker by single-positive (either CD4+ or CD8+) T cells in the periphery [6,8]. Although several investigations support that mature CD4+ T cells are the source of DPTs, there is also evidence that CD8+ T cells could also be the primary cellular type [6]. Unlike immature thymic DPTs, peripheral DPTs exhibit the functional properties of mature T cells, including antigen-dependent cytokine production, cytolytic activity, and expression of markers associated with the memory phenotype [9,10]. DPTs are divided into two main populations based on the differential expression of each marker (CD4^{high}CD8^{low} and CD4^{low}CD8^{high}) [1–3]. In healthy donors, CD4^{high}CD8^{low} cells have an effector or memory phenotype (TEM), whereas CD4^{low}CD8^{high} cells display a central memory phenotype (TCM), which can switch to an effector phenotype during viral infections such as hepatitis C virus (HCV) and HIV [9,10].

Little is known about the functionality of DPTs, though their function seems to be disease-specific. DPTs exhibit cytotoxic potential in chronic viral infections, such as lymphocytic choriomeningitis virus (LCMV) [11] and HIV [12], and in certain types of cancer [13–15]. DPTs can have a regulatory role in malignancies [13,14], systemic sclerosis [16], and inflammatory bowel disease [17]. In autoimmune diseases, DPTs can be found in different compartments; they increase in peripheral blood among patients with myasthenia gravis [18] but are found infiltrating the affected tissues in autoimmune thyroid disease and rheumatoid arthritis [19,20]. In systemic sclerosis and rheumatoid arthritis, DPTs secrete mainly IL-4 [16,19] whereas in tumors, such as melanoma and cutaneous lymphoma, the primary cytokine produced is TNF- [13,14]. In chronic parasitic infections, such as in Chagas disease, DPTs are not only increased in peripheral blood [5,21] but are also found infiltrating the cardiac tissue in patients with advanced Chagasic cardiomyopathy [22,23].

Due to the growing interest in the study of DPT subpopulations and their potential roles in specific diseases, it seems essential to determine reference values among healthy individuals. Therefore, the main goal of this study is to establish standard values of DPTs and to evaluate their functional profile by determining the presence of one specific activation marker in suitable donors from a blood bank in Bogotá, Colombia.

**Methods**

**Study and donors**

This is a descriptive and cross-sectional study of suitable donors who volunteered for blood donation during 2017 at the National Blood Bank Colombian Red Cross in Bogotá, Colombia. The protocol and informed consent was approved by the Ethical Committee of Universidad de los Andes (act 209 of 2013). One hundred and three donors were enrolled in this study and signed the informed consent. The demographic characteristics of our study population are shown in Table 1. Three of them were excluded due to the presence of reactive serological tests for syphilis. The study population included 55 men and 45 women that fulfilled the donation requirements and had negative screening tests [HIV, syphilis, hepatitis C virus, hepatitis B virus, Chagas disease and human T-cell lymphotropic virus (HTLV)]. They ranged from 19 to 61 years of age. Samples were obtained from citrate phosphate dextrose (CPD) anticoagulated blood bags and transported
refrigerated from the blood bank to the Biomedical Sciences Lab, where the cellular analyses were conducted.

**Cell labeling and cytometry acquisition**

One hundred µL blood samples were used for labeling. Antibodies included anti-CD3 APC (clone UCHT1), anti-CD4 PerCP (SK3), anti-CD8 FITC (SK1), and anti-CD154 PE (TRAP1). All antibodies were purchased from BD Pharmingen (BD, San Diego, CA). Blood was stained in darkness for 30 minutes at 4°C and then incubated with a cell lysis buffer (BD FACS Lysing Solution) for 15 minutes at room temperature. Subsequently, cells were washed twice in phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO) (0.01 M, pH 7.4, PBS 1X) and gently resuspended. Viability was assessed with 7-aminoactinomycin D staining (7-AAD, BD). Samples were acquired and analyzed in an FACS Canto II flow cytometer with FACSDiva 6.1 software (BD Bioscience, San Jose, CA). At least $5 \times 10^4$ cells were acquired in the CD3+ T lymphocyte population gate according to their forward scatter (FSC) and side scatter (SSC) features. The gating strategy for CD3+ T cells and DPTs is shown in Fig. 1.

**Statistical analysis**

Information about the donor characteristics is shown in percentages. The Shapiro-Wilk normality test was conducted for all data obtained from the cellular analysis. A nonparametric statistical analysis was performed in the study. The Mann-Whitney U test was used to compare the means between two groups. The Kruskal-Wallis test was used to compare the means among multiple groups. The results are shown as medians and interquartile ranges (IQR). Statistical analysis was performed using GraphPad Prism 7 software (San Diego, CA). Significance was established at $p<0.05$.

**Results**

T lymphocytes are a highly heterogeneous group of immune cells that have been of great interest in clinical and biomedical research studies. In an effort to establish values between the different subpopulations of DPTs, lymphocytes were analyzed from peripheral blood mononuclear cells (PBMCs) from each blood donor included in the study. Lymphocytes were gated according to FSC vs SSC features, as shown in Fig. 1a. The median cell viability was 99.15% (IQR, 98.8 – 99.4%) in all samples, as shown in Fig. 1b. CD3+ T cells were subsequently identified according to cell surface expression of CD4 or CD8, as shown in Fig. 1c and Fig. 1d, respectively. DPTs were classified into two main subpopulations: CD4$^{\text{high}}$CD8$^{\text{low}}$ and CD4$^{\text{low}}$CD8$^{\text{high}}$, as shown in Fig. 1d. The median total percentage of DPTs among CD3+ T cells in all samples studied was 2.6% (IQR, 1.7 – 3.67%), and the subpopulation CD4$^{\text{high}}$CD8$^{\text{low}}$ showed a median content of 1.15% (IQR, 0.8 – 2.0%), whereas in the subpopulation CD4$^{\text{low}}$CD8$^{\text{high}}$, it was 0.9% (IQR, 0.5 – 1.67%), as shown in Fig. 2A. CD4$^{\text{high}}$CD8$^{\text{low}}$ accounted for 57.97% of DPTs, as shown in Fig. 2B. Total DPTs were analyzed according to sex. Women showed a higher percentage of DPTs (median, 3.3%; IQR, 2.2 – 4.15) than men (median, 2.1%; IQR, 1.6 – 3.3), $p=0.007$, as shown in Fig. 3. The activation status of DPTs was assessed by using the surface marker expression of CD154, also called CD40L, as shown in Fig. 1E. The subpopulation of CD4$^{\text{low}}$CD8$^{\text{high}}$ showed higher expression of CD154 than the other T cell populations ($p \leq 0.0001$), as shown in Fig. 4.
Discussion
In recent decades, there has been growing interest in CD4+CD8+ double-positive T lymphocytes, which are considered a separate subpopulation of T cells associated with different pathologic conditions. In this study, a reference percentage value was established among DPT subpopulations. An activation marker was also studied in the blood samples of volunteer blood bank donors. An increased frequency of DPTs was found in women when compared to men. A sex variance has been found in other cell blood subpopulations, such as NK lymphocytes [24,25], and it would be of particular interest to evaluate DPTs during pregnancy and in placental tissue due to the sex difference found.

The frequency of DPTs in peripheral blood does not increase in HIV, HCV or melanoma; however, this subpopulation exhibited a higher expression of surface activation markers (i.e., HLA-DR and CD38) and greater cytokine production (i.e., IFN- and TNF-) in individuals with these diseases when compared to controls [7,9,10,14]. Nonetheless, in one study assessing HIV, an increased frequency of DPTs expressing CD38 and HLA-DR was associated with advanced disease in patients with a CD4+ count of <200 cells/uL [7], which are markers that have been widely used to define T cell activation by antigens [26]. Additionally, an increased frequency of DPTs in peripheral blood was found in chronic Chagasic patients [22,23] and among individuals with myasthenia gravis [18], and the percentage of DPTs interestingly decreased after treatment in both diseases [18,23]. Among patients with melanoma, there was an increased frequency of DPTs in draining lymphoid nodes and tumor infiltrating lymphocytes (TILs) [14].

In this study, a higher expression of CD154 (CD40L) was found in CD4lowCD8high cells. This activation marker has been used as an indicator for antigen-specific T cell activation in CD4+ T cells [27] and in CD8+ T cells [28]. Remarkably, this DPT subpopulation has an effector memory phenotype [9,10]. CD154 is the ligand of CD40, and this axis has been found to be of particular interest in the therapeutics of autoimmune diseases [29]. It would be very important to elucidate the functional role of CD154 in DPTs. Other markers have been studied on DPT cells including activation, homing, and differentiation markers [10,22]. Due to the role of DPTs in the pathogenesis of several diseases, it seems promising to study the expression inhibitory molecules such as PD-1 or CTLA-4. These molecules are currently targets of immunotherapy for different tumor conditions [30–32]. In previous reports, no other activation markers, such as CD38 and HLA-DR, were found in DPTs from healthy donors [7,10,22].

In this study, a median DPT of 2.6% was found, which was a higher result than that of the control donors in previous studies. For instance, in controls used to study DPTs in HIV patients, the median was 0.8% (IQR, 0.1 - 1.2) [7]; in melanoma, the mean was 0.9% (standard deviation SD 0.6) [14]; in hepatitis C virus infection, the mean was 1% (SD 0.6) [10], and in chronic Chagas disease, the mean was 1.1% (0.5) [22]. However, the control donors analyzed in these studies were from small cohorts, and demographic information about blood donor characteristics was lacking. Certainly, our study sample was significantly larger than those included in prior investigations, which could explain the increments evidenced in the results. Indeed, in one study, the frequency of DPTs ranged from 0-5% in control donors [18]. These findings and the differences found between the sexes can be used for future reference in specific populations and diseases. The limitations of this study include the age restriction of our sample and the limited screening tests.
performed in each donor. To the best of our knowledge, this is the first study to assess the frequency of DPTs in a large cohort of blood bank donors.

**Acknowledgements.** We would like to thank all the donors who volunteered to be a part of this study, the personnel of the National Blood Bank Colombian Red Cross who aided in the collection of the samples and Juan Guillermo Ripoll, MD, of Mayo Clinic for revising the manuscript. This project was performed and finalized in memory of Manuel Salamanca, who conceived, wrote and standardized the protocols.

**Conflict of interest.** The authors declare that they have no conflict of interest.
Table 1. Characteristics of the population studied.

<table>
<thead>
<tr>
<th>Ages</th>
<th>18-29</th>
<th>30-49</th>
<th>50-65</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women</td>
<td>11</td>
<td>18</td>
<td>16</td>
<td>45</td>
</tr>
<tr>
<td>Men</td>
<td>6</td>
<td>33</td>
<td>16</td>
<td>55</td>
</tr>
<tr>
<td>No.</td>
<td>17</td>
<td>51</td>
<td>32</td>
<td>100</td>
</tr>
<tr>
<td>Age average (±SD)</td>
<td>24.4 (3.5)</td>
<td>40.9 (6.4)</td>
<td>55.7 (3.0)</td>
<td>42.9 (11.8)</td>
</tr>
</tbody>
</table>

Age in years, SD: standard deviation
References

1. Zloza A, Al-harthi L. Multiple populations of T lymphocytes are distinguished by the level of CD4 and CD8 coexpression and require individual consideration. J Leukoc Biol. 2006;79:10–2.


Figures

Figure 1.

**Figure legend 1.** Flow cytometry gating strategy. (A) Peripheral blood mononuclear cells distributed in dot plot by flow cytometry according to FSC versus SSC. (B) Cell viability using 7-aminoactinomycin D. (C) T cells identified by the expression of CD3. (D) Dot plot distribution showing the expression of CD4 and CD8, and the gate on DPTs: CD4\textsuperscript{high}CD8\textsuperscript{low} and CD4\textsuperscript{low}CD8\textsuperscript{high}. (E) Density plot showing CD154 expression in each DPT subpopulation.
Figure legend 2. (A) Percentage of subpopulations of DPTs from the total T lymphocytes. (B) Percentage of subpopulations among the total DPTs. Figures are displayed as median with minimum and maximum.

Figure 3.
**Figure legend 3.** Percentage of the total DPTs according to sex. Women had higher percentages of DPT cells than men. Mann-Whitney, \( p=0.007 \). Figures are displayed as median with minimum and maximum.

**Figure 4.**
**Figure legend 4.** Expression of CD154 in single-positive and double-positive T cell subpopulations. The subpopulation CD4$^{low}$CD8$^{high}$ had higher CD154 expression than other subpopulations of T cells. Figures are displayed as median with minimum and maximum.

**Contributor’s Statement**

1. M.S.G.: Collection of the blood samples, cell labeling, flow cytometry analysis, statistical analysis, interpretation of findings, writing of the manuscript.
2. N.I.B.: Cell labeling, flow cytometry analysis, interpretation of findings.
3. M.S.: Design of the study, writing of the protocol.
6. J.M.G.: Flow cytometry analysis, statistical analysis, interpretation of findings, writing of the manuscript.