Viability and Functionality of Cryopreserved Peripheral Blood Mononuclear Cells in Pediatric Dengue

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Cryopreserved peripheral blood mononuclear cells (PBMCs) are widely used in studies of dengue. In this disease, elevated frequency of apoptotic PBMCs has been described, and molecules such as soluble tumor necrosis factor (TNF)-related apoptosis-inducing ligands (sTRAIL) are involved. This effect of dengue may affect the efficiency of PBMC cryopreservation. Here, we evaluate the viability (trypan blue dye exclusion and amine-reactive dye staining) and functionality (frequency of gamma interferon [IFN-γ]-producing T cells after polyclonal stimulation) of fresh and cryopreserved PBMCs from children with dengue (in acute and convalescence phases), children with other febrile illnesses, and healthy children as controls. Plasma sTRAIL levels were also evaluated. The frequencies of nonviable PBMCs detected by the two viability assays were positively correlated ($r = 0.74$; $P < 0.0001$). Cryopreservation particularly affected the PBMCs of children with dengue, who had a higher frequency of nonviable cells than healthy children and children with other febrile illnesses ($P \leq 0.02$), and PBMC viability levels were restored in the convalescent phase. In the acute phase, an increased frequency of CD3$^+$CD8$^+$ amine-positive cells was found before cryopreservation ($P = 0.01$). Except for B cells in the acute phase, cryopreservation usually did not affect the relative frequencies of viable PBMC subpopulations. Dengue infection reduced the frequency of IFN-γ-producing CD3$^+$ cells after stimulation compared with healthy controls and convalescent-phase patients ($P \leq 0.003$), and plasma sTRAIL correlated with this decreased frequency in dengue ($r = -0.56$; $P = 0.01$). Natural dengue infection in children can affect the viability and functionality of cryopreserved PBMCs.

Cryopreservation is the maintenance of cells and biological tissues at low temperatures and is based on the use of various media or solutions that form hydrogen bonds with water molecules, preventing cellular damage. The low temperatures allow the media or solutions that form hydrogen bonds with water molecules to devitrify during the acute phase (11). In some cases, the magnitude of cryopreservation is the maintenance of cells and biological tissues at low temperatures and is based on the use of various media or solutions that form hydrogen bonds with water molecules, preventing cellular damage. The low temperatures allow the media or solutions that form hydrogen bonds with water molecules to devitrify during the acute phase (11). In some cases, the magnitude of cryopreservation may affect viability, phenotype, and cellular functionality due to factors such as inadequate temperatures, the freezing protocol used, the expertise of the personnel, and freezing time (4, 5). The disease of the individual from whom the PBMCs come also affects cryopreservation. For example, PBMCs from subjects infected with human immunodeficiency virus (HIV) presented reduced viability after cryopreservation, possibly due to the increased number of apoptotic cells circulating during the course of the disease (6). Similar findings have been found in the acute phases of diseases, such as visceral leishmaniasis (7). Particularly for HIV, great efforts have been undertaken to optimize the evaluation and comparability of immune tests in cryopreserved PBMCs. Thus, studies evaluating the efficiency of cryopreservation of PBMCs from patients with particular diseases are greatly needed (8).

Dengue is another infectious disease in which an elevated frequency of PBMCs undergoing cellular death has been described (9). Dengue disease is caused by the dengue virus (DV), transmitted by mosquitoes of the genus Aedes, and constitutes a serious public health problem in tropical areas (10). A high frequency of apoptotic PBMCs, particularly CD8$^+$ T lymphocytes (TLs), circulate during the acute phase (11). In some cases, the magnitude of cellular death has been associated with clinically severe forms of disease (12). Although apoptosis induction in PBMCs in the context of dengue infection is a mechanism to control viral replication (13), the elevated frequencies of cells in different stages of cellular death may affect the efficiency with which these cells are cryopreserved. Knowling the efficiency of cryopreservation of PBMCs from children naturally infected with dengue is critical to certain studies, such as the search for cellular correlates of vaccine-induced protection. Here, we evaluate the viability and functionality of cryopreserved PBMCs from children naturally infected with DV (acute and convalescent), and these PBMCs were compared with those from healthy children or children who presented febrile pediatric infections other than dengue. The soluble form of the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (sTRAIL) in plasma was also determined to evaluate possible mechanisms associated with cellular dysfunction of cryopreserved PBMCs.

MATERIALS AND METHODS

Ethics statement. This study was approved by the Ethics Committee at the Universidad Surcolombiana (approval code NCS-047) and the Hospital Universitario de Neiva (approval code HUN-031). Written informed consent from parents and informed assent (for children older than 6 years) were obtained for each of the included children. All experiments followed the principles expressed in the Declaration of Helsinki.

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Patients and samples. This study was carried out in the Laboratorio de Infección e Inmunidad at Universidad Surcolombiana and the Hospital Universitario de Neiva, Colombia. Patients and healthy children were enrolled from February 2012 to January 2014.

Three groups of children, between 2 months and 14 years of age, were included in this study: healthy (n = 14), other febrile illnesses (OFI; n = 15), and infection with DV (n = 20). For the latter two groups, a blood sample in the acute phase (3 to 7 days from the onset of symptoms) was taken. Additionally, for the children with dengue, a second sample was taken 15 to 27 days from the onset of symptoms (convalescent dengue [CD]).

To two milliliters of venous blood was collected in tubes containing EDTA (product no. 367861; BD Vacutainer). Within the first 4 h after phlebotomy, the tubes were centrifuged at 300 × g, and the plasma was collected and stored at −70°C until the time of analysis. The cellular fraction was used for the isolation of PBMCs, as described below.

Diagnosis of DV infection. For the diagnosis, classification, and clinical monitoring of dengue patients, the revised guide of the World Health Organization (WHO) 2009 (14) was followed, which classifies the disease into dengue without warning signs (DNS), dengue with warning signs (DWS), and severe dengue (SD). The diagnosis of infection was confirmed by the presence of the viral nonstructural protein 1 (NS1) and/or DV-specific immunoglobulin M (IgM) in plasma (assessed before and after 5 days from the onset of symptoms, respectively). Children with OFI had diagnoses of bronchiolitis, common cold, group, or viral pharyngitis, in addition to negative tests for the dengue types mentioned above.

The commercial enzyme-linked immunosorbent assay (ELISA) kits Dengue IgM Capture (E-DEN01M), Dengue IgG Capture (E-DEN02G), and Dengue Early (E-DEN02P) were used for the detection of DV-specific plasma IgM and IgG and the viral protein NS1, respectively (all from Panbio; Alere, Australia), following the manufacturers’ instructions. For the type of infection (primary or secondary), the relationship of DV-specific IgM/IgG in the plasma was determined, taking a ratio of ≤2 as a secondary infection, as previously reported (15).

PBMC isolation and cryopreservation. The isolation, freezing, and thawing of PBMCs were performed as has been previously reported (16). Of note, this protocol has been frequently used (17–19). After isolation, the PBMCs were washed twice with RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine (complete medium) (all obtained from Gibco, Carlsbad, CA). For cryopreservation, the PBMCs were washed twice with complete medium, resuspended, and counted by trypan blue staining as explained below. Subsequently, the PBMCs were slowly resuspended in FBS containing 10% dimethyl sulfoxide (DMSO) (4× freezing medium; ATCC, Manassas, VA) precooled to 4°C at a cellular density of 5 × 10⁶ cells/ml and were deposited into polypropylene cryovials (product no. 375418; Nunc, Thermo Scientific, Waltham, MA) to be rapidly brought to −70°C in polystyrene containers, which ensured a slow drop in temperature. After 24 h, the cryovials were transferred to a liquid nitrogen tank (catalog no. CY509106; Thermo Scientific, Waltham, MA), where they remained until analysis. The cryopreservation time ranged from 4 to 120 weeks. For their thawing, the PBMCs were removed from the liquid nitrogen and were incubated in a preheated serological bath at 37°C. Once thawed, the cells were rapidly transferred to 15-ml polystyrene tubes (product no. 352099; Falcon-BD, San Jose, CA) containing 10 ml of cold complete medium to remove excess DMSO. Finally, the cells were washed with complete medium and counted. To calculate the percentage of recovery of PBMCs, the number of cells obtained was compared before and after cryopreservation using the following formula: (number of PBMCs after thawing/number of cryopreserved PBMCs) × 100.

In a fraction of experiments, PBMCs from healthy volunteers had cellular death induced by being frozen in excess DMSO (40% in FBS) and were used to standardize the evaluation methods of cell viability and as positive controls in the assays.

Determination of PBMC viability before and after cryopreservation. This study was designed to evaluate the total viability and not a particular type of cellular death. Cellular viability was evaluated using two methods: automated counting using trypan blue dye exclusion and staining of cellular amines by flow cytometry (FC). The trypan blue dye exclusion staining was performed following a widely used protocol (16). A 1:1 (vol/vol) mixture of PBMC suspension and 0.4% trypan blue (catalog no. 111732; Merck, Darmstadt, Germany) was incubated for 2 min at room temperature. Ten microliters of the mixture was deposited on 75- by 25- by 1.8-mm polymethyl methacrylate plates (catalog no. 145-00111, counting slides; Bio-Rad, Hercules, CA), and the plates were read in a TC20 automated cell counter (catalog no. 145-0102; Bio-Rad, Hercules, CA). The analysis was performed using TC20 data analyzer software (Bio-Rad, Hercules, CA), adjusting the cell size gate between 7 and 20 μm. To corroborate the results, in all experiments, one reading was also performed by conventional light microscopy. For this reading, 10 μl of the same mixture was deposited in a Neubauer chamber and was counted using a Nikon Eclipse E100 optical microscope (Nikon, Melville, NY). Counting was performed by two trained observers, and the result was reported as the mean value obtained by them. At least 40 cells were counted in each field (16).

To determine cellular viability by flow cytometry, a LIVE/DEAD fixable dead cell stain commercial kit (catalog no. L34955; Invitrogen, Waltham, MA) was used, following the recommendations of the manufacturer. For this assay, 1 × 10⁶ cells were washed and resuspended in 1 ml of sterile 1× Dulbecco’s phosphate-buffered saline (DPBS) (catalog no. 14190-144; Gibco, Carlsbad, CA), stained with 1 μl of fluorochrome reagent, and incubated for 30 min at 4°C while protected from light. After washing with sterile DPBS and centrifugation at 196 × g, 1 μl of Tri test (anti-human CD3, clone SK7, anti-CD4, clone SK3, and anti-CD8, clone SK1, labeled with peridinin chlorophyll protein complex [PerCP], fluorescein isothiocyanate [FITC], and phycoerythrin [PE], respectively; catalog no. 340298; BD, San Jose, CA) and 2 μl of anti-CD19–PE-Cy7 (clone HIB19, catalog no. 560728; BD, San Jose, CA) were added, and the solution was incubated for 30 min at 4°C while protected from light. Finally, the cells were washed with 3 ml of fluorescence-activated cell sorter (FACS) buffer (0.5% bovine serum albumin [BSA]) (catalog no. A7906; Sigma-Aldrich, St. Louis, MO) and 0.02% sodium azide [catalog no. 106688; Merck, Darmstadt, Germany] in 1× phosphate-buffered saline (PBS, filtered) and fixed with 1% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). The PBMCs were acquired on a FACSCanto II cytometer using FACS Diva v6.1.3 software (BD, San Jose, CA) within 1 h of completion of staining.

Evaluation of the functionality of cryopreserved PBMCs before and after cryopreservation. The functionality of the PBMCs was evaluated for the capacity of CD3⁺CD4⁺ and CD3⁺CD8⁺ cells to produce gamma interferon (IFN-γ) after being treated with polyclonal stimuli. For this assay, 1 × 10⁶ PBMCs/ml resuspended in complete medium were stimulated with 50 ng/ml of phorbol 12-myristate 13-acetate (PMA) (catalog no. P8139; Sigma-Aldrich, St. Louis, MO) and 500 ng/ml of ionomycin (catalog no. 10634; Sigma-Aldrich, St. Louis, MO) and were incubated for 10 h at 37°C in 5% CO₂, with the last h in the presence of 10 μg/ml of brefeldin A (catalog no. B7651; Sigma-Aldrich, St. Louis, MO). Then, the cells were washed and centrifuged at 196 × g, and 10 μl of Tri test was added (catalog no. 340298; BD, San Jose, CA). After 30 min of incubation at 4°C while protected from light, the cells were washed and permeabilized with 300 μl of Cytofix/Cytoperm (catalog no. 554722; BD, San Jose, CA) for 20 min at 4°C. Subsequently, intracellular staining was performed with anti-human IFN-γ labeled with allophycocyanin (APC) (clone 25723.11; catalog no. 341117; BD, San Jose, CA), incubating for 30 min at 4°C. Finally, the cells were washed twice with 1× Perm/Wash solution (catalog no. 554723; BD, San Jose, CA) and were acquired within 1 h of completion of staining.

Detection of sTRAIL in plasma. The plasma sTRAIL concentration was evaluated by ELISA (Quantikine Human TRAIL/TNFSF10, catalog
TABLE 1 Epidemiological and paraclinical characteristics of the children included

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Healthy (n = 14)</th>
<th>OFI (n = 15)</th>
<th>Dengue (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in months, median (range)</td>
<td>60 (13–144)</td>
<td>2</td>
<td>48 (7–88)</td>
</tr>
<tr>
<td>Gender, male, no. (%)</td>
<td>8 (57.1)</td>
<td>7 (46.6)</td>
<td>14 (70)</td>
</tr>
<tr>
<td>Illness day, median (range)</td>
<td>3 (3–6)</td>
<td>5 (3–7)</td>
<td></td>
</tr>
<tr>
<td>Primary infection, no. (%)</td>
<td>11 (55)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary infection, no. (%)</td>
<td>9 (45)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DWS, no. (%)</td>
<td>12 (60)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD, no. (%)</td>
<td>8 (40)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage hematocrit, median (range)</td>
<td>ND</td>
<td>32.2 (28.9–39.4)</td>
<td>34.1 (11.8–47)</td>
</tr>
<tr>
<td>Leukocytes, × 10³/µl, median (range)</td>
<td>ND</td>
<td>13.8 (9–17.9)</td>
<td>4 (1.1–9.3)</td>
</tr>
<tr>
<td>Platelets, × 10³/µl, median (range)</td>
<td>ND</td>
<td>360 (48–565)</td>
<td>58 (16–249)</td>
</tr>
</tbody>
</table>

a Kruskal-Wallis test, Dunn’s post hoc test.
b P < 0.0001 versus healthy children and children with dengue.
c Fisher test.
d Mann-Whitney test.
e ND, not determined.
f P ≤ 0.0007 versus OFI.

no. DTRL00; R & D Systems) following all of the manufacturer’s recommendations. The reported sensitivity of the test is 2.8 pg/ml. The correlation coefficient of the standard curve was >99%, and the duplicate variability was <10%. The mean optical density (OD at 450 nm) of the negative controls was 0.055. Calculating the concentration of sTRAIL was done by interpolation of the OD of the samples to a standard curve using 4-parameter logistic regression with GraphPad Prism software version 6.0.

Statistical analysis. The data are presented as medians and ranges. GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA) was used for the statistical analyses. The Mann-Whitney test was used to analyze two independent groups, the Wilcoxon test was used for paired data. To analyze more than two independent groups, the Kruskal-Wallis test was used. If the Kruskal-Wallis P value was < 0.05, Dunn’s multipletest was used according to each case. The degrees of correlation between variables were determined with the Pearson and Spearman tests. Fisher’s test was used for frequency analysis. In all cases, a P value of < 0.05 was taken as significant.

RESULTS

Patients included. This study included 14 healthy children, 15 children with OFI, and 20 children with dengue (18 of them matched to acute dengue [AD] and convalescent dengue [CD] phases). Children with dengue were classified clinically as DWS (n = 12) or SD (n = 8) (Table 1). As is known, bronchiolitis, common cold, croup, and viral pharyngitis are common in children <12 months old (20), which explains the lower median age of children with OFI than of healthy children and children with dengue (P < 0.0001, Dunn’s post hoc test). The children with OFI and dengue were included between the third and seventh days of fever. Children with dengue had lower leukocyte and platelet counts than children with OFI (Table 1). The medians (ranges) of aspartate aminotransferase (AST) in children with DWS and SD were 39 U/liter (34 to 88) and 138 U/liter (36 to 172), respectively (P = 0.01, Mann-Whitney test; data not shown). Alanine aminotransferase (ALT) was also significantly higher in children with SD than in children with DWS (P = 0.01, Mann-Whitney test; data not shown). In summary, the data presented in Table 1 support the adequate clinical classification of the groups included. Of note, the medians (ranges) of the time (weeks) of PBMCs cryopreservation were 25 (15 to 98), 49 (24 to 72), 95.5 (8 to 112), and 27 (4 to 96) for the healthy children and children with OFI, AD, and CD, respectively, with no significant differences between them (P = 0.1, Kruskal-Wallis test; data not shown).

The cellular viability assays that were used evaluated the same cellular population. Automated cellular counting methods using dye exclusion with trypan blue and staining of cellular amines were used to assess PBMC viability. The two methods detect increased permeability of the cell membrane as a viability marker and not a particular type of cellular death (21). Figure 1A shows the frequencies of nonviable PBMCs detected by the two methods in the patients. Comparable frequencies were detected using the two methods (r = 0.74; P < 0.0001, Pearson test), which demonstrates their capacity to identify the same cellular population. PBMCs were thawed and counted to determine the percentage of recovery with respect to the number of cells originally fro-

FIG 1 The methods for evaluating cellular viability were comparable. (A) Correlation between the percentages of nonviable PBMCs determined by trypan blue and amine staining. All children included in the study are shown. Pearson’s correlation, P value, and slope of the curve are shown. (B) Percentages of cell recovery. The medians and respective ranges are shown. ns, not statistically significant by the Kruskal-Wallis test.
zen. As shown in Fig. 1B, the median (range) recoveries were 83% (62% to 100%), 84% (70% to 99%), 83.5% (70% to 100%), and 87% (38% to 100%) for the healthy children and children with OFI, AD, and CD, respectively, with no differences between the groups (P = 0.7, Kruskal-Wallis test).

Pediatric DV infection affects the viability of cryopreserved PBMCs. To establish whether natural DV infection in children affects the viability of cryopreserved PBMCs, the frequencies of nonviable PBMCs before and after freezing were evaluated. As previously reported (22), increased frequencies of nonviable cells were found in PBMCs after cryopreservation in all of the groups analyzed (P ≤ 0.0001, Wilcoxon test) (Fig. 2A), confirming the effect of the process on cellular viability. The median (range) of nonviable PBMCs from healthy children was low (4.6% [1% to 19%]) after cryopreservation and comparable to that reported previously (5, 23), demonstrating the efficiency of the freezing protocol used here (Fig. 2A). After cryopreservation, there was a higher frequency of trypan-positive PBMCs in children with acute DV infection than in healthy children (P = 0.0002, Dunn’s post hoc test) (Fig. 2A). In convalescence, the values of trypan-positive cells were lower than in the acute phase (P = 0.0002, Dunn’s post hoc test) (Fig. 2A) and were similar to those found in healthy children (P > 0.05, Dunn’s post hoc test) (Fig. 2A). Higher levels of trypan-positive cells were also found in children with dengue than in those with OFI (P = 0.02, Dunn’s post hoc test) (Fig. 2A), suggesting that this effect could be virus specific. Comparable results were obtained by amine staining, which was performed simultaneously (data not shown). Cryopreservation particularly affected PBMCs from children with dengue, as the relationship of the frequency of dead cryopreserved cells/dead fresh cells was at least 2-fold higher than that found in the other groups (P < 0.0001, Dunn’s post hoc test) (Fig. 2A). Analysis of the viability of cryopreserved PBMCs between children with DWS and children with SD by amine-reactive dye and trypan blue staining showed no difference (P > 0.05, Dunn’s post hoc test) (Fig. 2B and data not shown). In summary, in children naturally infected with DV, there was a greater frequency of nonviable PBMCs after cryopreservation, indicating a greater lability to this process. The frequency was not associated with clinical severity.

Phenotype of nonviable PBMCs from children with dengue. For identifying the type of cell that dies during dengue infection, the differences in the frequencies of specific subpopulations of amine-positive PBMCs between the AD phase and the CD phase (AD/CD ratio) were analyzed by FC. Amine-positive CD3+ CD4−, CD3+ CD8+ (CD4+ and CD8+ T lymphocytes, respectively), CD3− CD19+ (B lymphocytes), and CD3− CD19− (non-T non-B cells) cells were analyzed according to the strategy shown in Fig. 3A. Consistent with the previous results (Fig. 2), all of the PBMC populations had ratios greater than 1, indicating that death was higher in the acute phase of infection (Fig. 3B and C). Before cryopreservation, the AD/CD ratio of amine-positive CD3+ CD8+ cells was higher than that of amine-positive CD3+ CD4+ cells (P = 0.01, Dunn’s post hoc test) (Fig. 3B). However, after the process, no differences in any of the subpopulations evaluated were observed (P = 0.3, Kruskal-Wallis test) (Fig. 3C). These results suggest that in fresh PBMCs, the CD8+ T lymphocytes are particularly susceptible to death during the acute phase of infection. After preservation, this susceptibility is similar in all PBMC subpopulations, a fact explained by the increase in the frequency of dead cells due to the preservation process.

Cryopreservation usually conserved the relative frequencies of PBMC subpopulations. Subsequently, we evaluated the effects of the infection and cryopreservation on the relative frequencies of living CD3+ CD4+ , CD3+ CD8+, CD3− CD19+, and CD3− CD19− cells. The relative frequencies of all of the analyzed populations were comparable between the acute and convalescent phases regardless of whether the cells were fresh or cryopreserved (P ≥ 0.09, Mann-Whitney test) (Fig. 4). Furthermore, the relative frequencies of subpopulations of PBMCs were usually not affected by the cryopreservation (Fig. 4), and only a lower frequency of viable CD19+ cells was found after cryopreservation compared with before cryopreservation in AD (P = 0.02, Mann-Whitney test) (Fig. 4C), indicating that this subpopulation may be more labile to the cryopreservation.
Therefore, cryopreservation generally maintains the relative frequencies of the different PBMC subpopulations in children infected with dengue.

Natural infection with DV affects the functionality of PBMCs. The capacity of the cryopreserved PBMCs from children with dengue to produce IFN-γ in response to stimulation with PMA-ionomycin was further evaluated. Consistent with previous reports (24, 25), high CD4 downregulation was found, so that after stimulation, the CD4+ T cells were analyzed as CD3+ CD8+ cells. In healthy controls, the medians (ranges) of IFN-γ-producing CD3+ CD4+ and CD3+ CD8+ T cells were 5.7% (1.2% to 12.2%) and 5.9% (2.7% to 13%), respectively (Fig. 5), frequencies consistent with previous reports (26). After cryopreservation, PBMCs from children with dengue had lower frequencies of IFN-γ-producing CD3+ CD8+ (Fig. 5A) and CD3+ CD8− T cells after stimulation (Fig. 5B) than PBMCs from healthy children (P = 0.003 and P = 0.0001, respectively; Dunn’s post hoc test). In convalescence, the frequencies of IFN-γ-producing CD4+ and CD8+ T cells were restored to levels comparable with those of the healthy, indicating that the IFN-γ downregulation was virus induced. Similar results were obtained before cryopreservation (data not shown). This effect is not dependent on the stimulus used, as similar results were observed when fresh PBMCs were treated with Staphylococcus aureus enterotoxin B, a known superantigen (n = 5; data not shown). Short protocols using PMA-ionomycin particularly stimulate memory T lymphocytes (26), which are low in infants (under 1 year of age) (27), and would explain the low frequency of IFN-γ-producing T cells.
also found in children with OFI (Fig. 5A and B). Of note, there was no association between the frequencies of IFN-γ-producing TLs after polyclonal stimulation, CD3⁺ CD8⁺ and CD3⁺ CD8⁻, and the clinical severity of DV infection (DWS versus SD, \( P \approx 0.4 \), Mann-Whitney test; data not shown). In summary, in acute-phase natural DV infection decreased the frequency of IFN-γ-producing TLs after in vitro stimulation, thus showing an effect on their functionality.

**Plasma sTRAIL correlates negatively with the low frequency of IFN-γ-producing T cells.** To explore mechanisms explaining the high frequency of nonviable cryopreserved PBMCs and the low functionality induced by natural DV infection in children, levels of sTRAIL, a molecule associated with cellular dysfunction, were evaluated by ELISA in children with dengue and OFI (in the sample of the same day that the PBMCs were cryopreserved). The medians (ranges) in picograms per milliliter for plasma sTRAIL were found to be significantly lower in children with DV infection compared to OFI and healthy controls (Fig. 6). The medians (ranges) were as follows: healthy (300–6000 pg/mL), OFI (50–1500 pg/mL), AD (20–400 pg/mL), and CD (20–400 pg/mL). The P value from the Mann-Whitney test is shown in each case.

**FIG 4** Cryopreservation generally maintains the relative frequency of the major PBMC subpopulations in children with dengue. Frequencies of amine-negative (amine⁻) CD3⁺ CD4⁺ (A), CD3⁺ CD8⁺ (B), CD3⁺ CD19⁺ (C), and CD3⁺ CD19⁻ (D) cells pre- \( (n=5) \) and post-cryopreservation \( (n=16) \) in the acute and convalescent phases of DV infection. The medians and their respective ranges are shown. ns, not statistically significant. The \( P \) value of the Mann-Whitney test is shown in each case.

**FIG 5** Natural infection with DV affects the functionality of cryopreserved PBMCs. Frequencies of cryopreserved IFN-γ-producing CD3⁺ CD8⁺ cells (A) and CD3⁺ CD8⁻ cells (B) after in vitro stimulation with PMA-ionomycin were analyzed by FC. The horizontal lines indicate the median for each group. ns, not statistically significant. The \( P \) value from Dunn’s post hoc test is shown in each case.
were 117 (14 to 253), 194 (87 to 314), and 113 (66 to 130) in children with OFI, DWS, and SD, respectively (Fig. 6A). Of note, children with SD had lower levels of sTRAIL than those of children with DWS (P = 0.01, Dunn’s post hoc test) (Fig. 6A). No correlation between the frequency of dead cells and the respective plasma sTRAIL levels in children with dengue or OFI was found (rho ≤ 0.2; P ≥ 0.4, Spearman test; data not shown). However, plasma sTRAIL correlated negatively and moderately with the low frequency of IFN-γ-producing CD3+ TLs (CD4+ and CD8+) in children with dengue (rho = −0.56; P = 0.01, Spearman test) (Fig. 6B) but not in children with OFI (rho = 0.4; P = 0.3, Spearman test; data not shown). These results suggest that soluble factors such as sTRAIL may be partially involved in the decreased functionality of TLs observed during acute DV infection.

**DISCUSSION**

In this study, the viability and functionality, before and after cryopreservation, of PBMCs from children with dengue were evaluated. (i) Cryopreserved PBMCs from children with dengue had a higher frequency of nonviable cells than those from healthy children or children with OFI. (ii) Before cryopreservation of PBMCs from children with dengue, the population with the highest frequency of dead cells was that of CD8+ TLs. (iii) Cryopreservation usually maintained the relative frequencies of PBMC subpopulations from children with dengue. (iv) Dengue virus infection in the acute phase reduced the frequency of IFN-γ-producing TLs after polyclonal stimulation, and this inhibition was associated with increased plasma sTRAIL levels.

The two methods that evaluated cellular viability had a strong positive correlation (Fig. 1A), and the percentages of recovery were similar between the study groups (Fig. 1B), suggesting that the majority of the cells, regardless of their viability, were analyzed, without significant cell loss during the freezing process. Consistent with what was previously reported (22, 28), cryopreservation affected PBMC viability in all of the groups studied (Fig. 2A). Due to dehydration, mechanical and chemical stress, intracellular crystallization, and thermal shock, the cryopreserved cells had reduced viability after the process (1); in cryopreserved PBMCs, frequencies of nonviable cells of 5% to 10%, such as obtained here, are generally accepted in samples of healthy individuals (28).

PBMCs from children with dengue had a higher frequency of death than those of healthy children; PBMC viability was restored in convalescence (Fig. 2A). The frequency of nonviable cells in children with dengue found here is consistent with previous reports that used propidium iodide and annexin V (29). The mechanisms by which DV induces cell death are not entirely clear and are dependent on the cellular type analyzed. These mechanisms include (i) accumulation of viral proteins (30), (ii) induction of the expression of CD137, a death receptor (31), and (iii) induction of cellular death directly by viral proteins (32). In the dengue group, frequencies of dead cells were comparable in children with or without antigenemia (positive detection of plasma NS1) (data not shown), suggesting that other mechanisms in addition to the cell viral infection are responsible for the higher death levels found in dengue PBMCs. Activation-induced apoptosis (activation-induced cell death [AICD]) would be a critical mechanism for PBMC death, as high expression levels of members of the TNF receptor superfamily classically associated with cell death, such as CD95 (FAS) and sTRAIL, in PBMCs and the plasma of patients with the infection have been shown (33–36). This mechanism modulates immune cell activation against the virus, ensuring homeostasis (37). PBMC death in acute DV infection has been linked to disease severity (11, 12). In our study, this association was not found (Fig. 2B), although it should be noted that patients with dengue without warning signs were not included, which may behave differently than the more severe hospitalized forms analyzed here.

Few studies have analyzed the PBMC subpopulations particularly affected by death in DV infection. Preceding cryopreservation, CD8+ TLs were those that died in the acute phase of the infection in particular (Fig. 3B). In fresh PBMCs from individuals with dengue, apoptotic antigen-specific CD8+ TLs have been detected (9), and this effect has been associated with AICD (11). Following the cryopreservation, all of the subpopulations were
infection, and these levels were lower in the severe cases (Fig. 6A). In general, cryopreservation maintained the relative frequencies of the principal viable subpopulations of PBMCs in children with dengue (Fig. 4). However, a decrease in the relative frequency of amine-negative CD19⁺ cells was found after cryopreservation in the acute phase of infection (Fig. 4C). As a rapid and strong response of antibody-secreting cells has been reported in the acute phase of infection (38) and these types of cells are susceptible to cellular death (39), cryopreservation may affect this population. Furthermore, studies analyzing the effects of cryopreservation on the expression of differentiation markers and the frequency of antigen-specific effector B cells from children with dengue are necessary.

Multiple studies have evaluated the cellular functionality following PBMC cryopreservation. In healthy individuals, the performance of functional tests that assess T and B memory cells by methods such as enzyme-linked immunosorbent spot assay (ELISPOT), after polyclonal stimulation, is usually adequate (40), but there is still controversy as to whether these findings are similar in PBMCs of the sick (41). As has been previously noted, the cryopreservation did not affect the functionality of cells from healthy children (Fig. 5). However, children with dengue had a low frequency of IFN-γ-producing TLs after polyclonal stimulation, a number that was restored in the convalescent phase (Fig. 5), suggesting an inhibitory effect of the virus on their functionality. DV can inhibit IFN-α production (42) but possibly not IFN-γ production, although other flaviviruses, such as West Nile virus, do inhibit the production of the two interferon types (43). However, the continuous activation of PBMCs during dengue infection may force the cells to a state of exhaustion (44, 45). In addition, an inhibitory effect of interleukin-10 (IL-10), a cytokine expressed during the infection, on the secretion of other cytokines should also be considered (46).

High levels of sTRAIL were found in children with acute DV infection, and these levels were lower in the severe cases (Fig. 6A). Previously, sTRAIL has been attributed to an antiviral mechanism in dengue, given by the induction of apoptosis or the production of type I IFN (47), reducing the cellular viral burden (35) and thus enabling the higher levels of sTRAIL found in children who did not develop severe forms of the infection (Fig. 6A) (36, 48). There was no correlation between plasma sTRAIL and the frequency of dead cells (data not shown); however, a negative correlation with the low frequency of IFN-γ–producing TLs was detected (Fig. 6B). Thus, sTRAIL may be implicated in mechanisms of functional T cell inhibition in dengue. Consistently, TRAIL can suppress the low frequency of IFN-γ-producing TLs was detected (Fig. 6B).

infection should be taken into account when employing cryopreserved PBMCs.

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