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 ≤ 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 ≤ 0.003), and plasma sTRAIL correlated with this decreased frequency in dengue (rho = −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 cells to enter a quiescent state in which cellular functions are suspended without affecting their intrinsic characteristics (1). Peripheral blood mononuclear cells (PBMCs) are frequently cryopreserved for use in transplants or immunological studies (2, 3). However, the cryopreservation process 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 numbers 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. Knowing 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 (OFl; 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 to 27 days from the onset of symptoms (convalescent dengue [CD]).

Two to four 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 (Ig) M in plasma (assessed before and after 5 days from the onset of symptoms, respectively). Children with OFI had diagnoses of bronchiolitis, common cold, croup, 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-DENO1M), Dengue IgG Capture (E-DENO2G), and Dengue Early (E-DENO2P) 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 methods: automated counting using trypan blue dye exclusion and staining. Particular care was taken to standardize the evaluation methods of cell viability and as positive controls in the assays.

PBMC viability 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. I045-0011, counting slides; Bio-Rad, Hercules, CA), and the plates were read in a TC20 automated cell counter (catalog no. I045-0102; 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 fluorescent reagent, and incubated for 30 min at 4°C while protected from light. After washing with sterile DPBS and centrifugation at 196 × g, 10 μl of Tttest (anti-human CD3, clone SK7, anti-CD4, clone SK4, 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 B1HIB19, 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 FACSCan 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 PMBCs/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. 10643; 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 Tretest 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. 354572; 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

| Characteristic               | Healthy (n = 14) | OFI (n = 15) | Dengue (n = 20) |
|-----------------------------|-----------------|-------------|-----------------|
| Age in months, median (range)
| 60 (13–144)           | 2            | 48 (7–88)    |
| Gender, male, no. (%)      | 8 (57.1)       | 7 (46.6)    | 14 (70)        |
| Illness day, median (range) | 3 (3–6)        | 5 (3–7)     |                 |
| Primary infection, no. (%) | 11 (55)        | 9 (45)      |                 |
| Secondary infection, no. (%)| 12 (60)        |             |                 |
| DWS, no. (%)               | 32.2 (28.9–39.4) | 13.8 (9–17.9) | 360 (48–565) |
| Percentage hematocrit, median (range) | 34.1 (11.8–47) | 4 (1.1–9.3) | 58 (16–249) |
| Leukocytes, $\times 10^9/\mu l$, median (range) | ND | ND | ND |
| Platelets, $\times 10^9/\mu l$, median (range) | ND | ND | ND |

$^{a}$Kruskal-Wallis test, Dunn’s post hoc test.
$^{b}$P < 0.0001 versus healthy children and children with dengue.
$^{c}$Mann-Whitney test.
$^{d}$ND, not determined.

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, bronchialitis, 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.
natural infection with DV affects the viability of cryopreserved PBMCs. (A) Frequencies of pre- and post-cryopreservation trypan-positive PBMCs in the groups analyzed. The delta (Δ) of the frequencies of post/pre-cryopreservation trypan-positive cells is shown at the top of each group (for statistical purposes, the percentages equal to zero were carried to one). (B) Frequencies of post-cryopreservation amine-positive cells evaluated in the two clinical groups of children with dengue and healthy controls. The median of each group and the P value of Dunn’s post hoc test are shown. *, P < 0.0001, Dunn’s post hoc test for AD versus healthy, OFI, and CD.

**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 evaluated by FC. Amine-positive CD3+CD4+ CD3+CD8+ (CD4+ and CD8+ TLs, 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+ TLs 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⁺ TLs were analyzed as CD3⁺ CD8⁻ cells. In healthy controls, the medians (ranges) of IFN-γ-producing CD4⁺ CD8⁻ and CD3⁺ CD8⁻ TLs were 5.7% (1.2% to 12.2%) and 5.9% (2.7% to 13%), respectively (Fig. 3), 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⁺ TLs 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 TLs.

**FIG 3** Dengue particularly affects the viability of fresh CD3⁺ CD8⁻ cells. (A) Gating strategy for analyzing the viability of cryopreserved PBMC subpopulations by FC in a child with dengue in the acute phase and in the convalescent phase. Relative frequencies of amine-positive (amine⁺) CD3⁺ CD4⁺, CD3⁺ CD8⁻, CD3⁺ CD19⁻, and CD3⁻ CD19⁻ cells (non-T non-B cells, bottom) are shown. (B, C) Ratios of the frequencies of amine⁺ CD3⁺ CD4⁺, CD3⁺ CD8⁻, CD3⁻ CD19⁻, and CD3⁻ CD19⁻ cells in the acute and the convalescent phase of infection by DV (AD/CD) before (n = 5) (B) and after (n = 16) (C) cryopreservation. The medians and ranges are shown. ns, not statistically significant. The P value of Dunn’s post hoc test is shown.
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 = 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 as follows: AD IgG−: 4.2 (0.4–5.1) and IgG+: 10.8 (6.5–15.9); CD IgG−: 0.6 (0.2–1) and IgG+: 1.2 (0.5–3.2). 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−CD19− 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.
FIG 6 sTRAIL is negatively associated with the frequency of IFN-γ-producing CD3+ cells after in vitro stimulation. (A) Plasma sTRAIL levels in children with other febrile illnesses (OFI) and with acute DV infection (dengue warning sign [DWS] and severe dengue [SD]) were evaluated by ELISA. The dashed line indicates the detection limit of the assay. The solid horizontal lines indicate the median. The *P* value of Dunn's post hoc test is shown. (B) Correlation between plasma sTRAIL levels in children with dengue and the frequencies of cryopreserved IFN-γ-producing CD3+ cells after treatment with PMA-ionomycin. The *P* value and Spearman rank correlation (rho) are displayed.

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). However, 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
affected similarly in the acute and convalescent phases (Fig. 3C), which is possibly due to the effects of the cryopreservation previously described.

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 immunoabsorbent 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). SARZOTTI-KELSEO M, NEEDHAM LK, ROUNTREE W, BAINBRIDGE J, GRAY CM, FISCU S, FERRARI G, STEVENS WS, STAGER SL, BINZ W, LOUZARO R, LONG KO, MOGHTOPO P, MOODY N, MACKAY M, KERKA M, MCPHILLION T, KIRCHHERR J, SODERBERG K, HAYNES BF, Denny TN. 2014. The Center for HIV/AIDS Vaccine Immunology (CHAVI) multi-site quality assurance program for cryopreserved human peripheral blood mononuclear cells. J Immunol Methods 409:21–30. http://dx.doi.org/10.1016/j.jim.2014.05.013.

SOFRENTOV I, MEWAN K, CROWE S, MARIER J, DAVIES R, SUUROENJ J, KURATITI D. 2012. Circulating angiogenic cells can be derived from cryopreserved peripheral blood mononuclear cells. PLoS One 7:e48067. http://dx.doi.org/10.1371/journal.pone.0048067.

NAZARPOUR R, ZABHI E, ALIJNIPOUR E, ABEDIAN Z, MEHDIZADEH H, RAHIMI F. 2012. Optimization of human peripheral blood mononuclear cells (PBMCs) cryopreservation. Int J Mol Cell Med 1:88–93.

VALERI CR, PIVACEK LE. 1996. Effects of the temperature, the duration of frozen storage, and the freezing container on in vitro measurements in human peripheral blood mononuclear cells. Transfusion 36:303–308. http://dx.doi.org/10.1046/j.1537-2995.1996.3649626141.x.

WEINBERG A, SONG LY, WILKINKING CL, FENTON T, HURAL J, LOUZARO R, FERRARI G, ETTER PE, BERRONG M, CANNIFF J, CARTER D, DAEFA OD, GARCIA A, GARRELS TL, GELMAN R, LAMBRECHT LK, PAHWA S, PILAKKA- KANHITKEL S, SHUGARTS DL, TUSTIN NB. 2010. Optimization of storage and shipment of cryopreserved peripheral blood mononuclear cells from HIV-infected and uninfected individuals for ELISPOT assays. J Immunol Methods 363:42–50. http://dx.doi.org/10.1016/j.jim.2010.09.032.

POTESTIO M, D’AGOSTINO P, ROMANO GC, MILANO S, FERRARO V, AGNINO A, DI BELLA G, CARUSO R, GAMBINO G, VITALE G, MANSUETO S, CILLARI E. 2004. CD4+CCR5 and CD4+CCR3 lymphocyte subset and monocyte apoptosis in patients with acute visceral leishmaniasis. Immunology 113:260–268. http://dx.doi.org/10.1111/j.1365-2956.2004.01948.x.

AZIZ N, MARGOLICJB, DETELS R, RINALDO CR, PHAIR J, JAMIESON BD, BUTCH AW. 2013. Value of a quality assessment program in optimizing cryopreservation of peripheral blood mononuclear cells in a multicenter study. Clin Vaccine Immunol 20:590–595. http://dx.doi.org/10.1128/CVI.00693-12.

MONGKOLSAPAYA J, DEJNIRATTISAI W, SU, VASANAWATHANA S, TANGHWORCHAIRUK D, CHAIRUNGRU A, SAWASDIVORN S, DUANGCHinda T, DONG T, ROWLAND-JONES S, YENCHITSOMANUS PT, MCMICHAEL A, MALASIT P, SCRETON C. 2003. Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. Nat Med 9:921–927. http://dx.doi.org/10.1038/nm887.

BHATT S, GETHING PW, BRADY OJ, MESSINA JP, FARLOW AW, MOYES CL, DRAKE JM, BROWNSTEIN JS, HOEN AG, SANKOH O, MYERS MF, GEORGE DB, JAECH H, MCGRAW J, SIMPSON CP, SCOTT TW, FARRAR JJ, HAY SI. 2013. The global distribution and burden of dengue. Nature 496:504–507. http://dx.doi.org/10.1038/nature12060.

MYINT KS, ENDY TP, MONGKORSIRICHAIKUL D, MANOMUTH C, KALAYANAROSJO S, VAUGHN DW, NISALAK A, GREEN S, ROTMAN AL, ENNIS FA, LIBRaty DH. 2006. Cellular immune activation in children with acute dengue virus infections is modulated by apoptosis. J Infect Dis 194:600–607. http://dx.doi.org/10.1086/506451.
12. Jaiven Y, Masrinoul P, Kalayanarooj S, Pulmanaushahuk R, Ubol S. 2009. Characteristics of dengue virus-infected peripheral blood mononuclear cell death that correlates with the severity of illness. Microbiol Immunol 53:442–450. http://dx.doi.org/10.1111/j.1348-0421.2009.00148.x.

13. Martins Sde T, Silveira GF, Alves LR, Duarte dos Santos CN, Bordignon J. 2012. Dendritic cell apoptosis and the pathogenesis of dengue. Mediat Inflamm 2012:257326. http://dx.doi.org/10.1155/2012/257326.

14. World Health Organization and the Special Programme for Research and Training in Tropical Diseases. 2009. Dengue: guidelines for diagnosis, treatment, prevention and control: new edition. World Health Organization, Geneva, Switzerland.

15. Innis BL, Nisalak A, Nimmanithiya S, Kusalerdchariya S, Chongsawadi V, Suntayakorn S, Puttisiri P, Hoke CH. 1989. An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. Am J Trop Med Hyg 38:245–249. http://dx.doi.org/10.4269/ajtmh.1989.38.2.245.

16. Wunsch JG, Joseph HR, Green T, Field JA, Wooters M, Kaufhold RM, JG, Joseph HR, Green T, Field JA, Wooters M, Kaufhold RM, JG, Joseph HR, Green T, Field JA, Wooters M, Kaufhold RM, JG, Joseph HR, Green T, Field JA, Wooters M, Kaufhold RM. 2015. Innate immunity to dengue virus replication in human hepatoma cells activates NF-kappaB which in turn induces apoptotic cell death. J Virol 71:3244–3249.

17. Nagila A, Netsawang J, Sirisawat C, Noisakran S, Mochang A, Yasamut U, Puttikhun C, Kasinrerk W, Malaisit P, Yenchitsomanus PT, Limjindaporn T. 2011. Role of CD137 signaling in dengue virus-mediated apoptosis. Biochem J 438:428–433. http://dx.doi.org/10.1042/BJ20110515.

18. Catteau A, Roue G, Yuste VJ, Susin SA, Despres P. 2003. Expression of dengue ApoptoM sequence results in disruption of mitochondrial potential and caspase activation. Biochimie 85:789–793. http://dx.doi.org/10.1016/S0300-9084(03)00139-1.

19. Green S, Pichyangkol S, Vaughn DW, Kalayanarooj S, Nimmanithiya S, Nisalak A, Kurane I, Rothman AL, Ennis FA. 1999. Early CD95 expression on peripheral blood lymphocytes from children with dengue hemorrhagic fever. J Infect Dis 180:1429–1435. http://dx.doi.org/10.1086/315072.

20. Torres-Carvalho A, Marinho CF, de Oliveira-Pinto LM, de Oliveira DB, Damasco PV, Cunha RV, de Souza LJ, de Azedo EL, Kubelka CF. 2014. Regulation of T lymphocyte apoptotic markers is associated to cell activation during the acute phase of dengue. Immunobiology 219:329–340. http://dx.doi.org/10.1016/j.imbio.2014.10.018.

21. Goenka P, Skjerven HO, Mikelsen IB. 2014. Acute bronchiolitis in infants, a review. Scand J Trauma Resusc Emerg Med 22:23. http://dx.doi.org/10.1186/1757-7241-22-23.

22. Patton K, Aslam S, Lin J, Yu L, Lambert S, Dawes G, Esser MT, Woo J, Janetzki S, Cherukuri A. 2014. Enzyme-linked immunosorbent assay for detection of human respiratory syncytial virus F protein-specific gamma interferon-producing T cells. Clin Vaccine Immunol 21:628–635. http://dx.doi.org/10.1128/CVI.00713-13.

23. Kleeboonong C, Li, Van HW, Margolick JB, Rinaldo CR, Phair JP, Giorgi JV. 1999. Viability and recovery of peripheral blood mononuclear cells cryopreserved for up to 12 years in a multicenter study. Clin Diag Lab Immunol 6:14–19.

24. Oymar K, Skjerven HO, Mikelsen IB. 2014. Acute bronchiolitis in infants, a review. Scand J Trauma Resusc Emerg Med 22:23. http://dx.doi.org/10.1186/1757-7241-22-23.

25. Petersen SM, Chatterjee PK, Lamoreaux L, Nguyen R, Ambrozak D, Koup RA, Roederer M. 2006. Amine reactive dyes: an effective tool to discriminate live and dead cells in polychromatic flow cytometry. J Immunol Methods 313:199–208. http://dx.doi.org/10.1016/j.jim.2006.04.007.

26. Wunsch M, Caspell R, Kuerten S, Lehmann PV, Sundararaman S. 2015. Serial measurements of apoptotic cell numbers provide better acceptance criterion for PBMC quality than a single measurement prior to the T cell assay. Cells 4:50–55. http://dx.doi.org/10.3390/cells4010050.

27. Germann A, Oh YJ, Schmidt T, Schon U, Zimmermann H, von Briesen H. 2013. Temperature fluctuations during deep temperature cryopreservation reduce PBMC recovery, viability and T-cell function. Cryobiology 67:193–200. http://dx.doi.org/10.1016/j.cryobiol.2013.06.012.

28. Baran J, Kowalczyk D, Ozog M, Zembala M. 2001. Three-color flow cytometry detection of intracellular cytokines in peripheral blood mononuclear cells: comparative analysis of phorbol myristate acetate-ionomycin and phytohemagglutinin stimulation. Clin Diag Lab Immunol 8:303–313. http://dx.doi.org/10.1128/CDLI8.2.303-313.2001.

29. Petersen CM, Christensen EI, Andresen BS, Moller BK. 1992. Internalization, lysosomal degradation and new synthesis of surface membrane glycoprotein CD4 in phorbol ester-activated T-lymphocytes and U-937 cells. Exp Cell Res 201:160–173. http://dx.doi.org/10.1016/0014-4827(92)90360-K.

30. Matula S, Seyfarth RM, Mountford AJ. 2014. Flow cytometry and kinetics of cytokines determined by intracellular staining using flow cytometry. J Immunol Methods 223:115–121. http://dx.doi.org/10.1016/j.jim.200022-1759(98)00200-2.

31. Goenka A, Kollmann TR. 2015. Development of immunity in early life. J Infect 71(Suppl 1):S12–S120. http://dx.doi.org/10.1016/j.jinf.2015.04.027.

32. Smith JG, Joseph HR, Green T, Field JA, Wooters M, Kaufhold RM, Antonello J, Caulfield MJ. 2007. Establishing acceptance criteria for cell-mediated immunity assays using frozen peripheral blood mononuclear cells stored under optimal and suboptimal conditions. Clin Vaccine Immunol 14:527–532. http://dx.doi.org/10.1128/CVI.00435-06.

33. Malavige GN, Huang LC, Salimi M, Gomes L, Jewewardana C, Jayaratne SD, Ogg GS. 2012. Cellular and cytokine correlates of severe dengue infection. PLoS One 7:e50387. http://dx.doi.org/10.1371/journal.pone.0050387.

34. Marianneau P, Cardona A, Edelman L, Deubel V, Despres P. 1997. Dengue virus replication in human hepatoma cells activates NF-kappaB which in turn induces apoptotic cell death. J Virol 71:3244–3249.
Souza L, Motta-Castro AR, da Cunha RV, Kubelka CF, Nogueira RM, de-Oliveira-Pinto LM. 2014. Apoptotic mediators in patients with severe and non-severe dengue from Brazil. J Med Virol 86:1437–1447. http://dx.doi.org/10.1002/jmv.23832.

49. Lehnert C, Weiswange M, Jeremias I, Bayer C, Grunert M, Debatin KM, Strauss G. 2014. TRAIL-receptor costimulation inhibits proximal TCR signaling and suppresses human T cell activation and proliferation. J Immunol 193:4021–4031. http://dx.doi.org/10.4049/jimmunol.1303242.

50. Morales JC, Ruiz-Magana MJ, Ruiz-Ruiz C. 2007. Regulation of the resistance to TRAIL-induced apoptosis in human primary T lymphocytes: role of NF-kappaB inhibition. Mol Immunol 44:2587–2597. http://dx.doi.org/10.1016/j.molimm.2006.12.015.