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MAJOR ARTICLE

Identification and Characterization at the Single-Cell Level of Cytokine-Producing Circulating Cells in Children With Dengue

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In this study, we identified, at the single-cell level, naturally induced cytokine-producing circulating cells (CPCCs) in children with dengue virus (DENV) infection ranging clinically from mild to severe disease. Tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) CPCCs were detected in children with primary or secondary acute dengue virus (DENV) infection, and the pattern of these cytokines was similar to that seen in the supernatant of cultured peripheral blood mononuclear cells and partially comparable to that found in plasma. Monocytes, B cells, and myeloid dendritic cells (mDCs) were the primary CPCCs detected, and the frequency of mDCs was significantly higher in severe disease. B cells isolated from children with dengue spontaneously secreted TNF-α, IL-6, and interleukin 10, and supernatants from cultures of purified B cells induced activation of allogeneic T cells, supporting an antibody-independent function of these cells during DENV infection. Thus, CPCCs could be a new immune parameter with potential use to evaluate pathogenesis in this infection.

Keywords. dengue; monocytes; B cells; IL-6; TNF-α.

Dengue is a viral vector-borne disease with a substantial burden worldwide, with an estimated 390 million infections and 25000 fatal cases occurring each year [1]. Dengue results from infection with any of 4 antigenically related dengue virus (DENV) serotypes that belong to the *Flavivirus* genus and are transmitted to humans, particularly through *Aedes aegypti* mosquitoes. In humans, a wide range of peripheral blood mononuclear cell (PBMC) subsets are targets of DENV infection, such as monocytes [2, 3], dendritic cells, and B cells (BCs) [4], as well as skin Langerhans cells [5], macrophages, hepatocytes, and endothelial cells [6] in peripheral tissues.

Hallmarks of dengue pathophysiology include vascular leakage and hemorrhage, which are partly explained by an increase of systemic cytokine expression [7, 8]. After exposure to DENV in vitro, several types of human cells secrete cytokines: interleukin (IL) 6, IL-10, and tumor necrosis factor alpha (TNF-α) are detected when monocytes/macrophages are infected [9]. Human umbilical vein endothelial cells supporting DENV replication secrete IL-6 and IL-8 [10]. Human BCs are susceptible to infection in vitro, and increased levels of IL-6 and TNF-α have been detected in these cultures [11]. However, studies analyzing the pattern and magnitude of cytokine expression in human primary cells naturally exposed to DENV during infection are less common. Increased expression of TNF-α and interferon gamma (IFN-γ) transcripts have been detected using quantitative polymerase chain reaction (PCR) or microarrays of bulk PBMCs obtained from patients with dengue, and the expression of TNF-α has been correlated with severe disease [12–15]. Additionally, monocytes isolated from children with dengue secrete detectable amounts of TNF-α, IL-1β, IL-12p70, and IL-17 [16].

We recently demonstrated that DENV infection in children induces dysfunction of particular subsets of PBMCs such as monocytes, plasmacytoid dendritic cells (pDCs), and T cells, as they produced lower levels of proinflammatory cytokines than those isolated from healthy children [17]. However, these results were obtained when PBMCs isolated from children with DENV infection were stimulated ex vivo with well-characterized stimuli.

Here, we determined the frequency, phenotype, and functional aspects of spontaneous CPCCs induced during natural DENV infection in children with mild and severe disease.

METHODS

Patients and Samples

The present study was approved by the Ethics Committee of the Universidad Surcolombiana (approval code NCS-047) and the Hospital Universitario de Neiva (approval code 2010–031).

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Written informed consent (from parents) and informed assent (from children >6 years of age) were obtained. All experiments followed the principles expressed in the Declaration of Helsinki. Seventy-three children with dengue and 34 healthy controls, between 4 months and 14 years old, were included in this study, which took place from January 2013 to April of 2015 in the Department of Huila, southern Colombia. For healthy children (control group), a pediatric examination and blood cell count were performed and children with any current disease were not included in the study. Children with abnormal examinations were remitted to the Pediatric Department of the Hospital Universitario de Neiva and an appropriate treatment was administered. From each child, 1–4 mL of venous blood (weight-adjusted for children aged <2 years) was collected in tubes containing ethylenediaminetetraacetic acid (BD Vacutainer, San Jose, California). Although for children with dengue, the blood samples were included in a wide range time in the acute phase of infection (2–8 days from the onset of symptoms), almost 70% of them were collected among 3–5 days of symptoms beginning. The blood samples were centrifuged at 300*g* for 10 minutes, and the plasma was collected and stored at −70ºC until further analysis. All experiments were performed in fresh cells within the first 6 hours of sample collection.

Clinical Classification and Diagnosis of DENV Infection

For the diagnosis, classification, and clinical monitoring of children with dengue, the revised guidelines of the World Health Organization 2009 [18] were followed. The guidelines clinically classify DENV-infected patients as dengue without warning signs (DNS), dengue with warning signs (DWS), and severe dengue (SD). The present study included 15, 35, and 23 children with DNS, DWS, and SD, respectively, and 34 healthy age-matched controls. Epidemiological, clinical, and laboratory characteristics of the study cohort are described in Supplementary Table 1 and the related article [17].

The DENV infection was confirmed by the presence of the viral nonstructural 1 (NS1) protein and/or DENV-specific immunoglobulin M (IgM) in plasma using the commercial enzyme-linked immunosorbent assay kits Dengue IgM Capture and Dengue Early (both from Panbio, Alere, Australia). To establish the type of infection (primary or secondary), the DENV-specific IgM/immunoglobulin G (IgG) ratio in plasma was calculated. The infecting serotype was determined in plasma by conventional reverse-transcription PCR (RT-PCR), as described previously [19]. Cases positive for DENV using RT-PCR were also defined as confirmed DENV infection, independent of the NS1 and/or DENV-specific IgM results.

Detection of Cytokine-Producing Circulating Cells and Soluble Cytokines by Flow Cytometry

A flow cytometry assay (FCA) was developed to detect the subsets of CPCCs in children with dengue [17]. A list of the antibodies and the respective fluorochromes used here is presented in Supplementary Table 2. PBMCs from children with dengue and healthy controls were isolated through Ficoll density gradient (Ficoll-Paque PLUS, GE Healthcare, Waukesha, Wisconsin). After isolation, the surface lineage and intracellular staining were applied as previously reported [17]. At least 300 000 T cells, 10 000 monocytes, and 2000 pDCs were acquired in each experiment. Fluorescence minus 1 controls were included in a set of experiments, and spillover was not evident (data not shown). In a fraction of experiments, PBMCs from children with dengue and healthy controls were cultured for 48–96 hours at 37°C, 5% carbon dioxide (CO_2), the supernatants were subsequently collected, and cytokines were measured as described below. PBMCs treated with 1.25 µg/mL of *Staphylococcus aureus* enterotoxin B superantigen were included as a positive control.

The levels of IL-2, IL-4, IL-6, IL-10, IL-17A, and TNF-α in cell culture supernatants and plasma were assessed by flow cytometry using a multiplex and a flex set bead-based assay (BD, San Jose, California) and following the manufacturer´s instructions.

B-Cell Isolation and Culture

B cells from children with dengue and healthy controls were isolated through positive magnetic cell sorting using microbeads labeled with antihuman CD19 (Miltenyi Biotec, Auburn, California). After purification, at least 90% of the cells were $CD19^+$ as determined using FCA in all experiments (data not shown). Typically, 2×10^5 to 1×10^6 purified BCs were cultured at a density of 2×10^6 cells/mL for 96 hours at 37°C, 5% CO_2, and the supernatants were collected and the cytokines were measured as described. The supernatants of purified BCs polyclonally stimulated with 10 µg/mL of anti-B cell receptor, 1 µg/mL of lipopolysaccharide, 5 µg/mL of CpG oligodeoxinucleotide 2006 (InvivoGen, San Diego, California), and 10 ng/mL of human recombinant IL-2 (R&D, Minneapolis, Minnesota) were also analyzed as positive controls.

Analysis of PBMC Activation Induced by Allogeneic B-Cell–Derived Factors

PBMCs from healthy children were incubated at a density of 2×10^6 cells/mL for 12 hours at 37°C, 5% CO_2 in the presence or absence of the pure supernatant of unstimulated cultured allogeneic purified BCs from children with dengue or healthy controls. As a positive control, we included PBMCs treated with supernatant of polyclonally stimulated allogeneic purified BCs from children with dengue (as described above). Subsequently, the cells were harvested, washed with 3 mL of Fluorescence Activated Cell Sorting (FACS) buffer (0.5% bovine serum albumin, 0.02% sodium azide [Merck Darmstadt, Germany] in 1× PBS, 0.2 µm filtered) and centrifuged at 196*g* for 10 minutes. Lineage antibody cocktails to detect monocytes, BCs, and T cells (described in Supplementary Table 2) were added plus

anti-human CD69 Fluorescein isothiocyanate (Clone FN50) and incubated for 30 minutes at 4°C, followed by washing and acquisition using a FACS Canto II (BD).

Statistical Analysis

GraphPad Prism 7.0 for Mac (GraphPad Software, La Jolla, California) software was used for the statistical analysis. The data are presented as medians and ranges. The Mann–Whitney test was used to analyze 2 independent groups. To analyze >2 independent groups, the Kruskal–Wallis test was initially used. If the Kruskal–Wallis *P* value was <.05, then Dunn multiple comparisons test was used. Correlations between variables were determined using the Spearman test. Fisher and χ^2 tests were used for frequency analysis. In all cases, a *P* value <.05 was considered significant.

RESULTS

PBMCs Spontaneously Produce Cytokines During Acute DENV infection

To determine whether naturally occurring DENV infection induces CPCCs and if they can be detected at a single-cell level through FCA, PBMCs from children with documented acute DENV infection and healthy controls were incubated for 12 hours in the presence of Brefeldin A, and the existence of DENV infection-induced CPCCs was assessed by intracellular cytokine staining. As shown in Figure 1, low but detectable frequencies of spontaneous CPCCs producing TNF-α, IL-6, and IL-10 were observed in children with dengue but not in healthy controls, with statistically significant differences between both groups in the case of TNF-α– and IL-6–producing cells ($P = .002$ and $P = .01$, respectively, Mann–Whitney test; Figure 1A and 1B) and a tendency in the case of IL-10–producing cells ($P = .05$, Mann-Whitney test; Figure 1C). We did not detect natural DENV infection–induced cells producing IFN-γ, interferon alpha (IFN-α), IL-4, or IL-17A (data not shown). High levels of plasma IL-6 and IL-10 (but not TNF-α, likely due to the type of assay and sample used, as previously noted [20]) were present during DENV infection in those same children (Supplementary Figure 1) [17]. However, there was no correlation between the levels of any evaluated cytokines in plasma and the frequencies

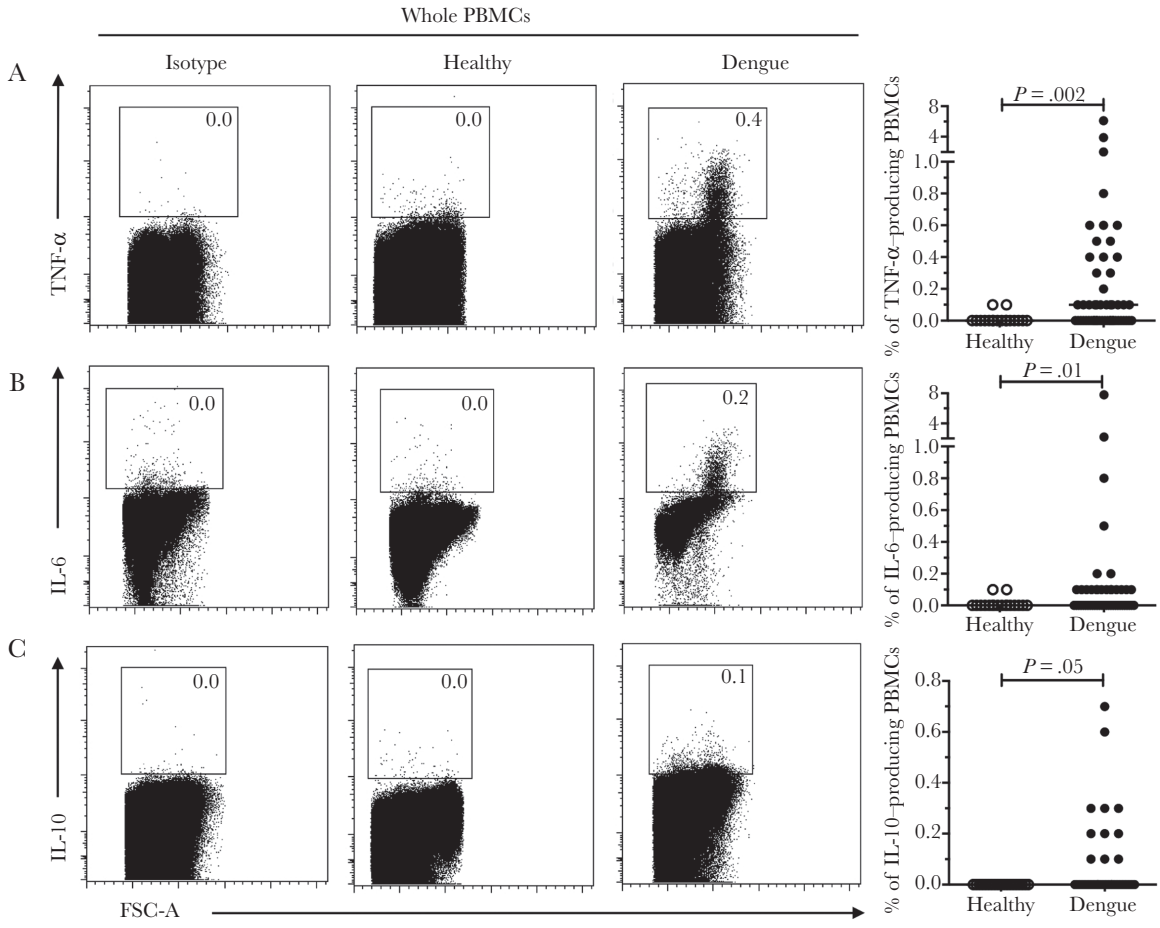

Figure 1. Cytokine-producing circulating cells (CPCCs) induced during natural dengue virus infection are detectable by flow cytometry. The frequencies of tumor necrosis factor alpha (*A*), interleukin (IL) 6 (*B*), and IL-10 (*C*) CPCCs in children with acute dengue and healthy controls were determined using flow cytometry. The dot plots of a representative child with dengue and a healthy control, and the isotype controls for each evaluated cytokine, are shown. A summary of all the patients and healthy children analyzed is shown on the right. The continuous lines indicate the median in each group. Mann–Whitney *P* values are shown. Abbreviations: FSC-A, Forward Scatter-A; IL-6, interleukin 6; IL-10, interleukin 10; PMBCs, peripheral blood mononuclear cells; TNF-α, tumor necrosis factor alpha.

of their respective CPCCs in children with dengue ($\rho \leq 0.2$, $P \geq .1$, Spearman test; data not shown). Similarly, the frequencies or detection of CPCC were not associated with the specific infecting serotype ($P \geq .1$, Dunn post hoc test), the presence or absence of circulating virus ($NS1^+$ or $viremia^+$ vs $NS1^-/viremia^-$; $P \geq .3$, Mann–Whitney test), the type of infection (primary vs secondary infection; $P \geq .2$, Mann–Whitney test), or the illness day (<4 vs ≥4 day; $P \geq .9$, Fisher test) (data not shown).

To confirm the FCA results, whole PBMCs from children with dengue and healthy controls were cultured for 48–96 hours, and the levels of cytokines in the supernatants were determined. As shown in Figure 2, higher levels of IL-6 and TNF-α were observed in supernatants of cultured PBMCs from children with dengue compared with healthy controls ($P = .004$ and $P = .006$, Mann–Whitney test, respectively), whereas there were no differences in the levels of IL-2, IL-4, IL-10, or IL-17A ($P \geq .1$, Mann–Whitney test). Thus, DENV infection in children induces detectable frequencies of naturally induced CPCCs expressing a particular pattern of cytokines, similar to the respective cytokines produced in culture supernatants, and partially similar to that found in their plasma (Supplementary Figure 1).

Monocytes, BCs, and Myeloid Dendritic Cells Are the Principal CPCCs Induced by Natural DENV Infection

Next, we characterized the subsets of CPCCs in peripheral blood of DENV-infected children. As shown in Figure 3 and Supplementary Figure 2, TNF-α–, IL-6–, and IL-10–producing monocytes, BCs, and myeloid dendritic cells (mDCs) were detected in children with dengue. Except for IL-10–producing mDCs, the frequencies of the 3 cytokine-producing subsets were higher in children with dengue than in healthy controls ($P \leq .01$, Mann–Whitney test; Figure 3). DENV infection–induced cytokine-producing T cells and pDCs were not detected (data not shown). In summary, after in vivo acute DENV infection in children, the principal subsets of CPCC are monocytes, BCs, and mDCs, with TNF-α and IL-6 being mainly produced by monocytes, BCs, and mDCs, and IL-10 mainly by monocytes and BCs.

Cytokine-Producing mDCs Are Associated With Severe Dengue

Some plasma cytokines have been associated with the clinical severity of dengue [21]. As monocytes, BCs, and mDCs detectable through FCA spontaneously produced cytokines during natural DENV infection, we hypothesized that their frequencies

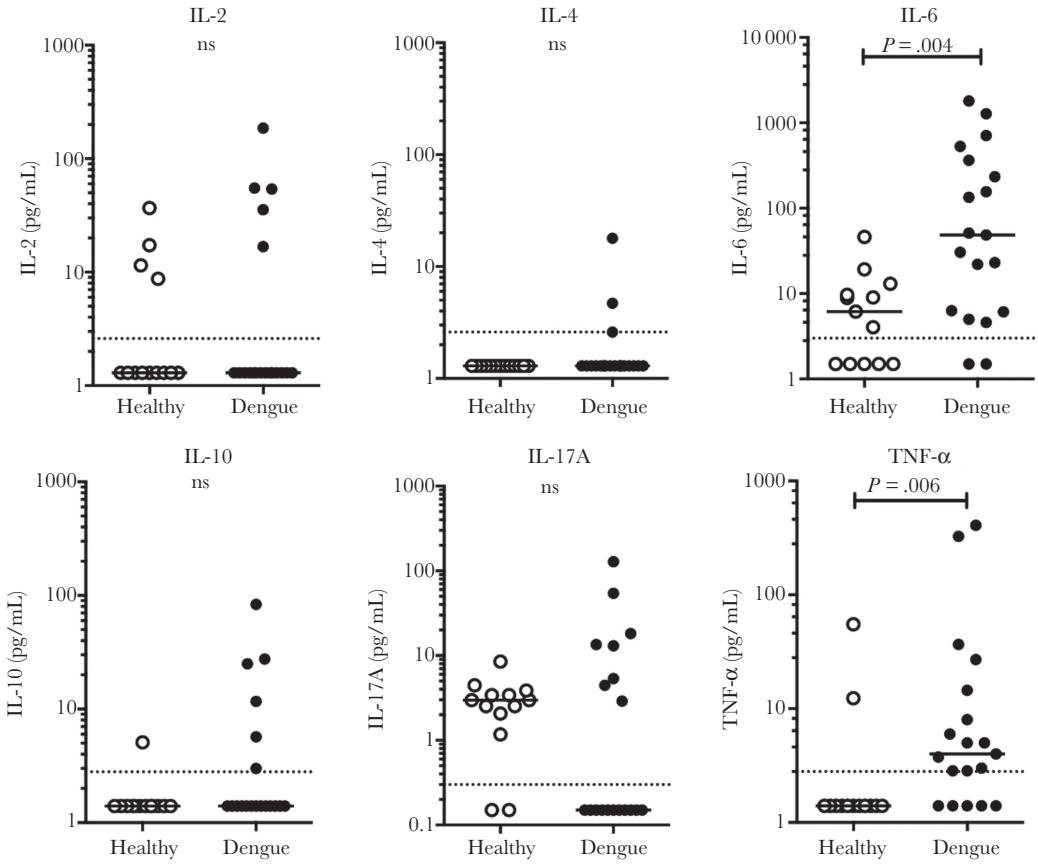

Figure 2. Peripheral blood mononuclear cells (PBMCs) from children with dengue spontaneously secrete cytokines. The levels of interleukin (IL) 2, IL-4, IL-6, IL-10, IL-17A, and tumor necrosis factor alpha (TNF-α) were evaluated by bead-based assays in supernatants from unstimulated cultured PBMCs isolated from children with dengue and healthy controls. The continuous lines indicate the median in each group. The dashed lines indicate the limit of detection of the assay for each cytokine. Mann–Whitney *P* values are shown (ns = not statistically significant).

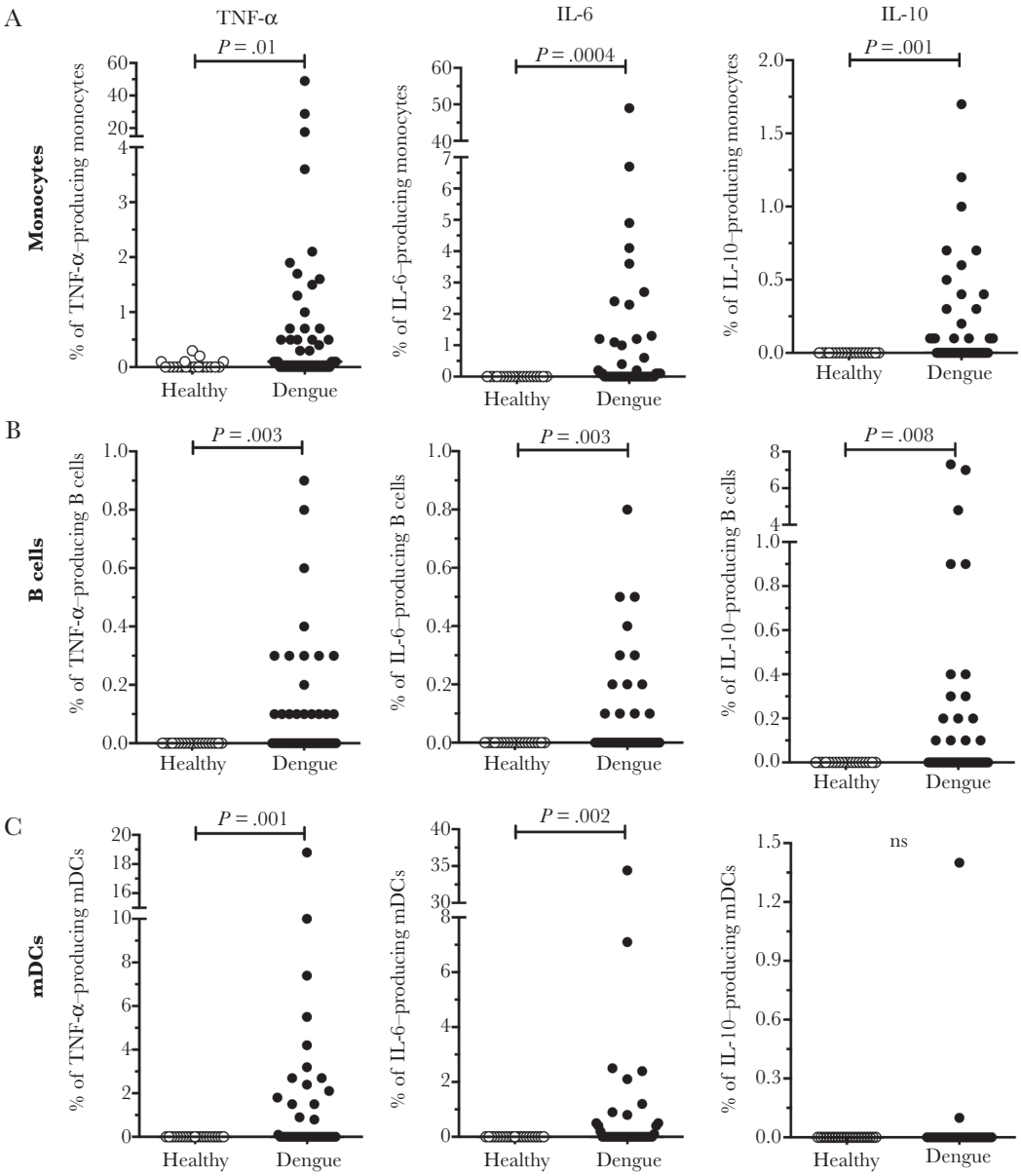

Figure 3. Monocytes (*A*), B cells (*B*), and myeloid dendritic cells (mDCs; *C*) were the principal subsets of cytokine-producing circulating cells (CPCCs) induced during dengue virus natural infection. The frequencies of tumor necrosis factor alpha (TNF-α)–, interleukin (IL) 6–, and IL-10–producing monocytes (*A*), B cells (*B*), and mDCs (*C*) in children with dengue and healthy controls were evaluated using flow cytometry. The continuous lines indicate the median in each group. Mann–Whitney *P* values are shown (ns = not statistically significant).

would be associated with clinical severity in children. As shown in Figure 4, higher frequencies of TNF-α– and IL-6–producing mDCs were observed in children with SD compared with children with milder dengue symptoms (DNS + DWS) ($P = .001$ and $P < .0001$, respectively, Dunn post hoc test). There were no differences in the frequencies of cytokine-producing monocytes and BCs between the groups of children with dengue ($P \geq .2$, Kruskal–Wallis test, data not shown). Thus, TNF-α– and IL-6–producing mDCs are positively associated with clinical severity in DENV-infected children.

Purified BCs From Children With Dengue Spontaneously Produce Cytokines and BC-Derived Factors That Induce the Activation of T Cells

As expected, monocytes and mDCs were detected as CPCCs during natural DENV infection, as these antigen-presenting cells are major targets of infection for this virus [22, 23]. Nevertheless, cytokine-producing BCs were also detected in children with dengue, suggesting a potential antibody-independent function for BCs during this disease. To further confirm and expand these findings, we isolated BCs from children with dengue and healthy controls, incubated them for 96 hours, and

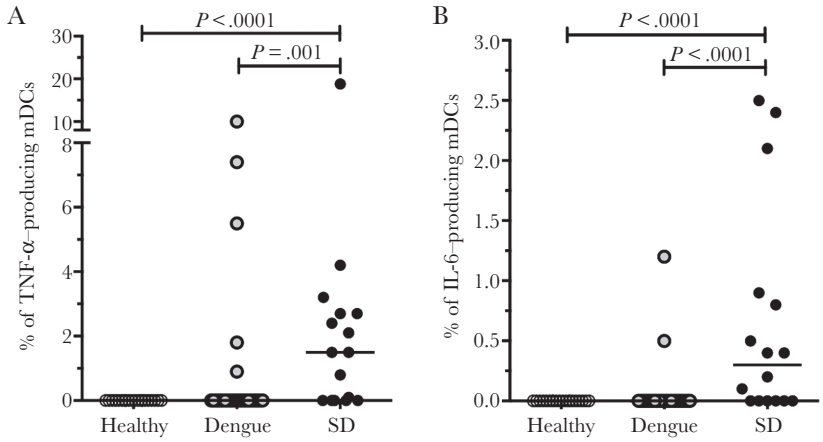

Figure 4. The frequencies of cytokine-producing myeloid dendritic cells (mDCs) are associated with clinical severity in dengue. Frequencies of tumor necrosis factor alpha (TNF-α)– (*A*) or interleukin 6 (IL-6)–producing mDCs (*B*) in children with dengue (dengue with no warning signs + dengue with warning signs), severe dengue (SD), and healthy controls were evaluated by flow cytometry. The continuous lines indicate the median in each group. Kruskal–Wallis and Dunn post hoc test *P* values are shown.

then measured cytokines in the culture supernatants. As shown in Figure 5, and in accordance with the FCA results (Figure 3B), higher levels of IL-6 and IL-10 (and a tendency in the case of TNF-α) were detected in the supernatants of purified BCs isolated from children with dengue compared with the healthy controls (P = .002, P = .02, and P = .06, respectively, Mann–Whitney test; Figure 5). There were no differences in the levels of IL-2, IL-4, and IFN-γ ($P \geq .7$, Mann–Whitney test, data not shown), excluding T-cell contamination in the BC suspension.

To evaluate the potential function of these naturally induced BC-derived factors during DENV infection, PBMCs from healthy children were incubated with supernatants of unstimulated allogeneic purified BCs isolated from children with dengue or healthy controls. The supernatant from polyclonally activated BCs was used as a positive control. The expression of CD69 (a well-known early cell activation marker) was assessed on the surface of T cells, monocytes, and BCs. As shown in Figure 6, a higher frequency of $CD3^+CD69^+$ T cells (P = .03, Mann–Whitney test; n = 5) was seen after 12 hours of incubation with supernatants from unstimulated allogeneic purified BCs from children with dengue than with those from healthy controls. In addition, the treatment of PBMCs with supernatants from unstimulated allogeneic purified BCs from children with dengue, but not from healthy control children, induced higher CD69 expression than that with medium alone (P = .01 and P = .6, respectively, Mann–Whitney test, Figure 6B). As expected, the expression of CD69 was higher on the T cells when the PBMCs were incubated with supernatants from polyclonally stimulated allogeneic purified BCs compared with the unstimulated conditions ($P \leq .02$, Dunn post hoc test; n = 5; Figure 6B). There was no increase in the expression of CD69 on monocytes and BCs under these conditions. In summary,

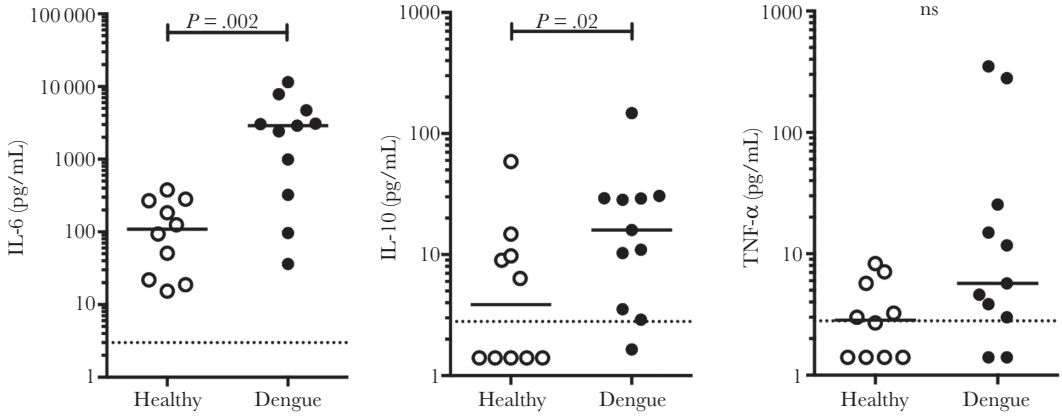

Figure 5. Purified B cells from children with dengue spontaneously secrete cytokines. The levels of cytokines were measured by bead-based assays in supernatant from unstimulated cultured positively purified B cells from children with dengue and healthy controls. The continuous lines indicate the median in each group. The dashed lines indicate the limit of detection of the assay for each cytokine. Mann–Whitney *P* values are shown. Abbreviations: IL-6, interleukin 6; IL-10, interleukin 10; ns, not statistically significant; TNF-α, tumor necrosis factor alpha.

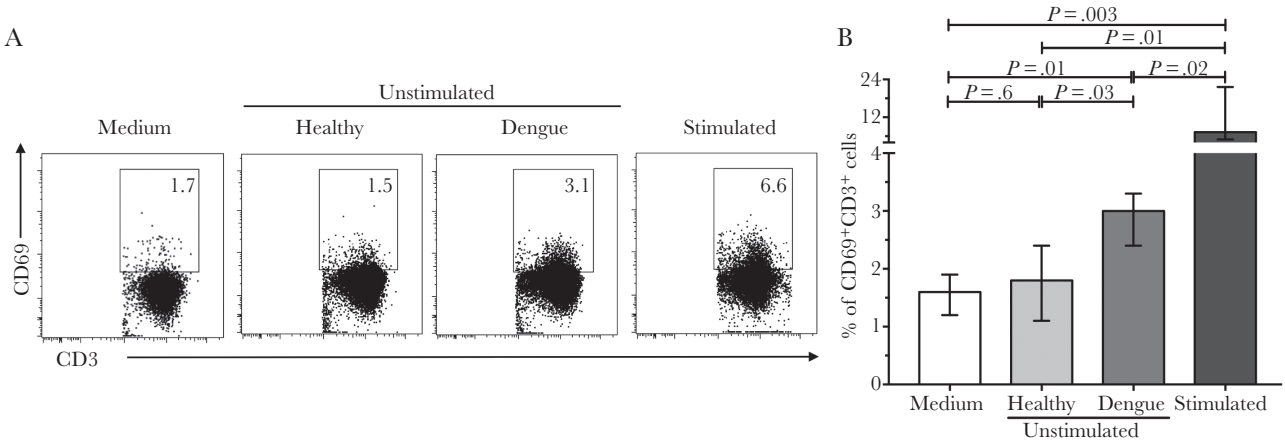

Figure 6. B cell–derived factors induce the activation of allogeneic T cells during natural dengue virus infection. *A*, Peripheral blood mononuclear cells from healthy children were incubated for 12 hours in the presence of supernatant of unstimulated cultured allogeneic purified B cells from children with dengue and healthy controls, supernatant of polyclonal stimulated B cells, or culture medium alone, and the frequency of $CD69^+CD3^+$ T cells was evaluated by flow cytometry. The dot plots of a representative child from a total of 5 children are shown. *B*, Summary of the frequency of $CD69^+CD3^+$ cells in all of the individuals included. Mann–Whitney *P* values are shown.

soluble factors derived from human BCs isolated from DENV-infected children induced the activation of T cells.

DISCUSSION

Here we analyzed the cellular cytokine response induced by natural DENV infection in children, observing that (1) PBMCs spontaneously produce a particular pattern of cytokines similar to that observed in culture supernatants of PBMCs and partially comparable to the cytokine pattern found in plasma of dengue-infected children; (2) monocytes, BCs, and mDCs constitute the major detectable CPCCs; (3) the frequencies of cytokine-producing mDCs are positively associated with dengue clinical severity; and (4) purified BCs spontaneously secrete cytokines and BC-derived factors that induce the activation of human T cells.

Vascular leakage and hemorrhage observed in dengue are partly explained by an increase of systemic cytokine expression [7, 8]. A particular pattern of plasma cytokines was observed in children with dengue (Supplementary Figure 1) [17]; however, the origin of plasma cytokines in DENV infection is unclear. Previously, PBMCs expressing TNF-α, TNF-β, IFN-γ, IL-2, IL-4, or IL-8 were observed in DENV-infected patients using RT-PCR and immunocytochemical staining [13–15]. Here, we present the first evidence of a low frequency of flow cytometry detectable CPCCs that produce IL-6, TNF-α, and likely IL-10 in children during dengue (Figure 1). This pattern was partially comparable to the cytokine pattern observed in plasma (Supplementary Figure 1) [17], and similar to that found in culture supernatants (Figure 2), indicating that PBMCs are probably a source of the circulating cytokines in dengue. However, the frequency of CPCC and the levels of the respective cytokine did not correlate, suggesting that some of the cytokine-producing cells that contribute to plasma cytokines levels likely migrated from blood to local tissues and have a different kinetics, or that other noncirculating cell types, such as endothelial cells [24], also contribute to the plasma cytokine levels in children.

Since in humans a wide range of PBMC subsets are targets of DENV infection [3, 4, 22], it is expected that PBMCs respond to DENV infection by producing cytokines. However, the particular circulating subsets responsible for these plasma cytokines are still undefined. Identifying these circulating subsets could be useful for developing new treatment approaches or new immune correlates of protection. Through single-cell analysis, cytokine-producing monocytes, BCs, and mDCs were detected in children naturally infected with DENV (Figure 3). Accordingly, purified monocytes from patients with dengue were found to secrete TNF-α, IL-6, and IL-12 p70 after 5 days in culture [16]. Also, these results are consistent with previous reports where monocytes, mDCs, and BCs produced a comparable pattern of cytokines after in vitro treatment with the DENV [11, 25, 26].

T cells and pDCs were undetectable as CPCCs. This finding is consistent, with the fact that DENV infection induces a virus-specific T-cell response with a peak on 7–14 days after fever onset [27, 28], which is out of range of the time points analyzed here. The blockage of type I IFN production by DENV [29] and, potentially, T-cell suppression and apoptosis [30] may also account for the failure to detect certain PBMC populations as cytokine producers. The few CPCCs naturally induced that were observed here, and the lower production of proinflammatory cytokines and proliferation of PBMCs isolated from dengue than those from healthy children after ex vivo stimulation [17, 31], could be 2 related events, as the DENV infection could induce cell exhaustion or increase in the expression of modulatory molecules such as the programmed cell death protein 1 in antigen-specific T cells [32].

When the clinical outcome was analyzed in this pediatric cohort, only a high frequency of proinflammatory

cytokine-producing mDCs was found to associate with severe forms of the dengue disease (Figure 4). Therefore, these activated professional antigen-presenting cells could be a useful new severity biomarker in pediatric dengue, although more prospective studies analyzing several time points during the acute phase would be necessary to fully establish this. This result is complementary to our previous findings that total and E protein–specific IgM antibody-secreting cells (ASCs) in infants and IgG ASCs in older children were associated with clinically severe forms of infection [33], and that antigenemia and infected circulating monocytes were associated with coagulation disorders [22], suggesting that severity of dengue is multifactorial.

Previously, it was reported that primary human BCs can be infected in vitro, polyclonally activated by DENV infection, and produce TNF-α and IL-6 [11, 34]. Furthermore, an in vivo expansion of circulating plasmablasts in patients with DENV infection has been reported [35, 36]. Other soluble factors implicated in T-cell activation and human immunity, such as TNF-superfamily members, can also be released by naturally activated BCs during DENV infection [37]. However, it had not been shown that BCs, at the single-cell level, express cytokines during natural DENV infection. Here, we observed that BCs from children with dengue spontaneously secrete a particular pattern of cytokines (Figures 3 and 5) that induce the activation of allogeneic human T cells (Figure 6). Although we did not determine which BC-derived factors were responsible for the activation of allogeneic T cells, the profile of cytokines encountered suggests that TNF-α and IL-6 could be involved in this effect [38, 39]. Additionally, the high levels of IL-10 suggest a potential regulatory role for BC during this viral infection, which merits further exploration. Nonetheless, apparently, the proinflammatory effect of TNF-α and IL-6 overcame the anti-inflammatory action of IL-10 under these evaluated conditions. The subpopulations of BCs that produce these cytokines remain unknown, but plasmablasts are a potential candidate, because of their massive expansion during acute DENV infection and capacity to secrete cytokines [40]. The CPCC kinetics and the antibody-independent functions of BCs in DENV infection deserve further investigation to better understand the pathogenesis of this disease and develop new strategies for treatment and prevention.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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