Virology 507 (2017) 11–19

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Selective dysfunction of subsets of peripheral blood mononuclear cells during pediatric dengue and its relationship with clinical outcome

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ARTICLE INFO

Keywords:
Dengue
Monocytes
T cells
Plasmacytoid dendritic cell
Cytokine
Interferon

ABSTRACT

During dengue virus (DENV) infection, a blockage of secretion of cytokines such as tumor necrosis factor (TNF)-α and members of the interferon (IFN) family has been described *in vitro*. We evaluated the functionality of monocytes as well as dendritic, B and T cells isolated from children with mild and severe dengue. Compared with those of healthy children, stimulated monocytes, $CD4^+$ T cells and dendritic cells from children with dengue had lower production of proinflammatory cytokines. The interferon axis was dramatically modulated by infection as plasmacytoid dendritic cells (pDCs) and $CD4^+$ T cells had low production of IFN-α and IFN-γ, respectively; plasma levels of IFN-α and IFN-γ were lower in severely ill children, suggesting a protective role. Patients with antigenemia had the highest levels of IFN-α in plasma but the lowest frequency of IFN-α-producing pDCs, suggesting that DENV infection stimulates a systemic type I IFN response but affects the pDCs function.

1. Introduction

Dengue is caused by any of four interrelated serotypes (dengue virus [DENV] 1–4) that belong to the *Flavivirus* genus and constitutes an important viral vector-borne disease causing an estimated 390 million infections around the world each year (Bhatt et al., 2013). Clinically, mild DENV infection is characterized by fever, skin rash, headache, arthralgia and myalgia. Vascular leakage, hemorrhage and organ dysfunction are hallmarks of severe forms of the disease (Olagnier et al., 2016). The increase of soluble factors such as interleukins (IL)-6, 8, 10 and 12p70, tumor necrosis factor (TNF)-α, and interferons (IFN)-α and γ partially explain these manifestations, and associations among several cytokines, and severe disease have been reported (Rothman, 2011).

DENV infection has classically been associated with an important immune activation. Involvement of the immune system is necessary for clearance and long-lasting protection against the virus; however, the DENV has developed specific mechanisms to subvert the antiviral immunity (Green et al., 2014). For instance, the secretion of IFN-α and β by primary human dendritic cells is inhibited by DENV *in vitro* infection (Rodriguez-Madoz et al., 2010), an effect explained by the blockage of pattern-recognition receptors (PRRs) or their signaling proteins, resulting in low type I IFN production (Gack and Diamond, 2016). In addition, the low quantities of IFNs produced do not have a relevant functional effect, as non-structural viral proteins (NS) 2A, 4A, 4B and 5 block the IFN receptor signaling pathway targeting the signal transducer and activator of transcription (STAT)1 and 2 proteins (Ashour et al., 2009; Munoz-Jordan et al., 2005). The nuclear factor-κB activation triggered by Toll-like receptor ligands is also blocked *in vitro* by DENV-2 infection, decreasing the secretion of some inflammatory cytokines such as TNF-α and IL-8 (Chang et al., 2012). Furthermore, subneutralizing antibodies amplify the burden of the cellular DENV infection, attenuate the production of IL-12 and IFN-γ and increase immunosuppressive mediators such as IL-10 (Ubol et al., 2010). These mechanisms could be related with the low cytokine production observed after *ex vivo* stimulation of PBMCs (Suharti et al., 2003; Torres et al., 2013; Pichyangkul et al., 2003), but their role in clinical outcome is still unclear.

Through *ex vivo* stimulation, intracellular cytokine staining and flow cytometry, we analyzed the functionality of the major peripheral blood mononuclear cells (PBMCs) subsets during natural DENV infection in children. Additionally, the levels of respective circulating cytokines were also determined. Monocytes, myeloid dendritic cells (mDCs), plasmacytoid dendritic cells (pDCs) and $CD4^+$ T cells from children with dengue had a limited production of cytokines such as TNF-α, IL-6 and IL-10. pDCs and $CD4^+$ T cells also had a low expression of IFN-α and IFN-γ, respectively, and the plasma levels of these cytokines were inversely associated with severe forms of the disease.

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http://dx.doi.org/10.1016/j.virol.2017.04.004
Received 28 February 2017; Received in revised form 3 April 2017; Accepted 4 April 2017
Available online 07 April 2017
0042-6822/

2. Methods

2.1. Patients and samples

This study was approved by the Ethics Committee of the Hospital Universitario de Neiva (code 031-22-12) and performed from July 2013 to August 2015 in the Department of Huila, southern Colombia, an endemic dengue area. All experiments followed the principles expressed in the Declaration of Helsinki. Children with dengue and healthy controls between 4 months and 14 years old were included in this study after their parents signed informed consent and assent (the latter in individuals older than 6 years of age). A pediatric assessment was done by the pediatrics department of the Hospital Universitario de Neiva in healthy children and any child with current disease was not included and the appropriated treatment was instated. Additionally, a complete blood cell count was obtained in all healthy children. From each child, 2–4 mL of venous blood (weight adjusted) was collected in tubes containing ethylenediaminetetraacetic acid (EDTA, BD Vacutainer®; Ref: 367861). For children with dengue, a single blood sample was collected in the acute phase of the infection (2–8 days from the fever onset). The tubes were centrifuged at 300 *x g*, and the plasma was collected and stored at −70 °C until the time of analysis. All cellular experiments were performed within the first six hours of the sample collection. Due to limited sample volume, some of the children were not included in all the analysis.

2.2. Diagnosis and classification of primary or secondary DENV infection

The revised dengue guidelines of the World Health Organization 2009 were followed for the diagnosis, classification, and clinical monitoring of dengue patients (Dengue, 2009). Accordingly, children were classified as having dengue without warning signs (DNS), dengue with warning signs (DWS) or severe dengue (SD). Children with DNS received non-hospital treatment and the other two groups (DWS and SD) received hospital-based management. The diagnosis of infection was confirmed by the presence of the viral NS1 protein and/or DENV-specific immunoglobulin (Ig) M in plasma (assessed before and five days after the onset of symptoms, respectively) using the commercial enzyme-linked immunosorbent assay kits Dengue Early (Ref: E-DEN02P) and Dengue IgM Capture (Ref: E-DEN01M), respectively (both from Panbio®, Alere, Australia). To establish the type of infection (primary or secondary), the DENV-specific IgM/IgG ratio was determined (the latter evaluated with the Dengue IgG Capture kit [Ref: E-DEN02G, Panbio®, Alere, Australia]), with a ratio of ≤2 considered a secondary infection (Toro et al., 2016). The current infecting serotype was determined by conventional reverse transcription polymerase chain reaction, as previously described (Lanciotti et al., 1992).

Children with DNS received non-hospital treatment (based in adequate oral rehydration and anti-pyretic intake) and the other two groups (DWS and SD) received hospital-based management (admitted to a secondary health care center for close monitoring of the hemodynamic state [daily blood cell count and the continuous assessment of vital signs, peripheral perfusion, urine output and organ function] and intravenous fluid therapy with isotonic solutions).

2.3. Antibodies for flow cytometry analysis

We used cell lineage antibody cocktails to analyze T cells, monocytes, B cells, mDCs and pDCs. For T cell detection we used anti-human CD3 APC-H7 (Clone SK7; Cat: 560176), anti-human CD4 V500 (Clone RPA-T4; Cat: 560768) and anti-human CD8 PerCP-Cy5.5 (Clone SK1, Cat: 341051) (all from BD, San Jose, CA); B cells and monocytes were identified using anti-human CD19 V500 (Clone HIB19, Cat: 561121) or CD19 FITC (Clone SJ25C1, Cat: 340409), anti-human CD20 APC-Cy7 (Clone L27, Cat: 335794) and anti-human CD14 PerCP-Cy5.5 (Clone M5E2, Cat: 550787) (all from BD, San Jose, CA); to detect mDCs and pDCs the Lineage cocktail 1 (CD3, CD14, CD16, CD19, CD20 and CD56; Cat: 340546), anti-human HLA-DR V500 (Clone: G46-6, Cat: 561224), anti-human CD123 PerCP-Cy5.5 (Clone 7G3, Cat: 558714) (all from BD, San Jose, CA) and anti-human CD11c APC-Cy7 (Biolegend, San Diego, CA, Clone: Bu15; Cat: 337217) were used. The following anti-human cytokine antibodies were used in appropriate doses and combinations for each cell lineage for the intracellular staining: anti-human IFN-γ FITC (Clone 25723.11, Cat: 340449), anti-human TNF-α PE-Cy7 (Clone Mab11, Cat: 557647), anti-human IL-10 APC (Clone JES3-19F1, Cat: 562036), anti-human IL-6 V450 (Clone MQ2-13A5, Cat: 561446) and anti-human IFN-α PE (Clone 7N4-1, Cat: 560097) (all from BD, San Jose, CA). Preliminary experiments were performed to determine the optimal doses of each antibody (n=3, data not shown).

2.4. Ex vivo *stimulation and detection of cytokine-producing cells*

PBMCs from children with dengue and healthy controls were isolated from whole venous blood using a Ficoll density gradient (Ficoll-Paque PLUS, GE Healthcare, Waukesha, WI; Cat: 17–1440-02). After isolation, the PBMCs were washed twice with RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 2 mM L-glutamine, 10 mM HEPES buffer, 1 mM sodium pyruvate and 0.1 mM MEM non-essential amino acids (all obtained from Gibco®, Carlsbad, CA, Cats: 11875-085, 16000-044, 10378-016, 15630, 11360 and 11140) and counted by Trypan blue staining (Merck, Darmstadt, Germany; Cat: 111732). Then, 2×10^6 cells/mL were separately stimulated with 1.25 μg/mL of *Staphylococcus aureus* Enterotoxin B (SEB, Sigma-Aldrich®, St. Louis, MO; Cat: S4881 [for stimulation of T cells]), 5 μg/mL of $F(ab')_2$ fragment goat anti-human IgA, IgG and IgM (Anti-B cell receptor [BCR], Jackson ImmunoResearch®, West Grove, PA; Code: 109-006-064 [for stimulation of B cells]), 1 μg/mL of *Escherichia coli strain O111:B4* lipopolysaccharide (LPS, Sigma-Aldrich®, St. Louis, MO; Cat: L2630 [for stimulation of monocytes, B cells and mDCs]) and 10 μg/mL of CpG ODN 2216 (Invivogen, San Diego, CA: Cat: tlrl-2006 [for stimulation of pDCs]) and incubated for 12 h at 37 °C in 5% CO_2, the last 9 h in the presence of 10 μg/mL of Brefeldin A (BFA, Sigma-Aldrich®, St. Louis, MO; Cat: B7651). Unstimulated cells were included as controls. After incubation and to increase cell recovery, the PBMCs were harvested with 1 mL of 1X Dulbecco's phosphate-buffered saline (DPBS, Gibco®, Carlsbad, CA; Ref: 14190-144), 0.5% Bovine Serum Albumin (BSA, Sigma-Aldrich®, St. Louis, MO; Cat: A7906), 2 mM EDTA (Gibco®, Carlsbad, CA; Ref: 15575-038) and washed with 3 mL of FACS buffer (0.5% BSA, 0.02% sodium azide [Merck, Darmstadt, Germany; Cat: 106688] in 1X PBS, filtered). Afterwards, lineage antibody cocktails for cell surface staining were added and incubated for 30 min at 4 °C, followed by a wash and cell permeabilization with 300 μL of Cytofix/Cytoperm (BD, San Jose, CA; Cat: 554722) for 20 min at 4 °C. Subsequently, 10 μL of FcR blocking reagent (Miltenyi Biotec, Auburn, CA; Order no: 130-059-901) was added and incubated 10 min at room temperature. Then, intracellular cytokine staining was performed, incubating for 30 min at 4 °C. Intracellular staining with appropriate isotype control antibodies was also performed. Finally, the cells were washed twice with 1X Perm/Wash solution (BD, San Jose, CA; Cat: 554723) and acquired on a FACS Canto II cytometer using FACS Diva v6.1.3 software (BD, San Jose, CA) within an hour of completing the staining. At least 2,000 pDCs (the least frequent subset within PBMCs evaluated) were acquired. Fluorescence minus one (FMO) controls were included in a set of experiments and spillover was not evident (data not shown).

2.5. Measurement of cytokines levels in plasma

The Cytometric Bead Array Human Th1/Th2 Cytokine kit II (BD,