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

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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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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,

San Jose, CA; Cat: 551809) and Human IFN-α Flex Set kits (BD, San Jose, CA; Cat: 560379) were used to measure IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IFN-α in plasma, following manufacturer's protocols. Samples were acquired within an hour following the procedure. The detection limits (in pg/mL) for IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IFN-α were 2.6, 2.6, 3, 2.8, 2.8, 7.1 and 1.5, respectively. The FACS Diva (v. 6.1.3) and FCAP (v. 3.0.14) software, both from BD, were used for bead acquisition and calculation of cytokine levels, respectively. Samples were analyzed in duplicate and a variability < 10% was noted; the mean of the duplicates for each sample is shown. Only experiments with standard curves with $R^2 \geq 0.98$ and significant P values for all cytokines evaluated were included.

2.6. Statistical analysis

GraphPad Prism® 7.0 for Mac (GraphPad Software, La Jolla, CA) software was used for the statistical analysis. The data are presented as medians and ranges and non-parametric analyses were performed. Mann-Whitney test was used for comparison of independent data. To analyze more than two independent groups, Kruskal-Wallis test was used. If the Kruskal-Wallis P value was < 0.05, then Dunn's multiple comparison test was used. The degree of correlation between variables was determined with the Spearman test. Fisher's and Chi Square tests were used for frequency analysis. In all cases, a P value < 0.05 was considered significant.

3. Results

3.1. Clinical and laboratory characteristics of the children included

We included 73 children with dengue (15 DNS, 35 DWS and 23 SD) and 34 healthy controls in this study. Table 1 describes the clinical and laboratory characteristics of our cohort. There were no differences in the age or gender of the groups (P≥0.1, Dunn's post hoc test). Children with DWS and SD were enrolled in a later day of illness than children with DNS (P≤0.005, Dunn's post hoc test) as the severe manifestations of dengue are present in this period. Twenty-three patients (31.5%) had primary DENV infections, with no differences observed between the frequency of primary or secondary infections in the three clinical severity grades (P=0.1, Fisher test). The most frequently identified serotype was DENV-3, followed by DENV-2 and DENV-1 (in the 61%, 32% and 4% of the typeable patients, respectively), according to the reports from Colombia during the period of the study (Ministerio de Salud y Protección Social, 2015). In the 35% of the patients it was not possible to determine the infecting serotype. As expected, children with DWS and SD but not with DNS had significant hepatomegaly and pleural effusion (P≤0.001, Dunn's post hoc test), and overall, the children with dengue had fewer white blood cells and platelet counts than healthy controls (P≤0.005 Mann-Whitney test). In addition, children with SD had higher aspartate and alanine transaminase levels than children with DWS (P=0.004 and P=0.02, Mann-Whitney test, respectively). It is noteworthy that, in our cohort, 13 (56.5%) of the children with SD in the late phase of the disease (after the 9th day of illness and at least 48 h after the sample collection) developed hospital-acquired infections including pneumonia (n=3) and urinary or gastro-intestinal sepsis (n=10), while none of the children with DNS or DWS presented with these complications.

3.2. DENV natural infection affects the ex vivo production of inflammatory cytokines by subsets of PBMCs

PBMCs from children with dengue and healthy controls were treated *ex vivo* with well-characterized stimuli and, through intracellular cytokine staining and flow cytometry (following the gating strategy of Supplementary Fig. 1), the frequencies of TNF-α, IL-10 and IL-6-producing monocytes, mDCs, pDCs, T cells and B cells were determined. The frequencies of all the cytokine-producing subsets after stimulation were significantly higher than in the unstimulated conditions, both in children with dengue and in healthy controls (P≤0.002, Man-Whitney test, data not shown), confirming the effects of the stimulation. The cytokine profile was as expected with the highest production of TNF-α, IL-10 and IL-6 by monocytes, followed by mDCs (Bueno et al., 2001). As shown in Fig. 1, compared to healthy controls, children with dengue had lower frequencies of TNF-α-producing monocytes, mDCs, pDCs and $CD4^+$ T cells (P≤0.04, Mann-Whitney test), of IL-10-producing monocytes and pDCs (P≤0.02, Mann-Whitney test, Fig. 1) and of IL-6-producing monocytes and pDCs (P=0.002, Mann-Whitney test, Fig. 1). The production of these cytokines by B cells and $CD8^+$ T cells from children with dengue was not affected compared to healthy controls (P≥0.6, Mann-Whitney test, data not shown). As expected, we did not detect IL-6 producing $CD4^+$ T cells (Villiger et al., 1991). The low frequencies of cytokine-producing monocytes, mDCs, pDCs and $CD4^+$ T cells were not due to decreased relative frequencies of these cell populations (after 12 h of incubation without any stimulus) in children with dengue compared to healthy controls, as they were unchanged in the case of monocytes (P=0.225, Mann-Whitney test, Table 2) and increased in the case of $CD4^+$ T cells, mDCs and pDCs (P=0.03, P=0.02 and P=0.005, respectively, Mann-Whitney test, Table 2).

The frequencies of cytokine-producing PBMC populations among the children with dengue were not related to the age of the patients (< 12 months vs. ≥12 months, P≥0.4, Mann-Whitney test, data not shown), indicating that the differences found here are not due to possible immaturity of the immune system. Similarly, frequencies of IL-6, TNF-α, or IL-10-producing PBMC subsets were not related to the infecting serotype (P≥0.2, Kruskal-Wallis test), the type of the infection (primary vs. secondary, P≥0.3, Mann-Whitney test), the presence or absence of circulating virus ($NS1^+$ and/or $Viremia^+$ vs. $NS1^-$ and $Viremia^-$, $P > 0.2$, Mann-Whitney test), the clinical severity (P≥0.4, Kruskal-Wallis test) or the day of illness (< 4 days vs. ≥4 days, P≥0.8, Mann-Whitney test) (data not shown).

3.3. DENV natural infection affects the production of type I and II Interferons

Considering that the IFN axis is critical for antiviral responses (Schneider et al., 2014) and some mechanisms of subversion of the IFN production pathway by DENV have been described *in vitro* (Morrison et al., 2012), the production of IFN-α and IFN-γ by pDCs and T cells (the major *in vivo* cellular source of IFNs) after stimulation with optimized doses of CpG ODN 2216 or SEB, respectively, was also evaluated. Children with dengue had dramatically lower frequencies of IFN-α-producing pDCs (Fig. 2A) and IFN-γ-producing $CD4^+$ T cells (Fig. 2B) compared to healthy controls ($P < 0.0001$ and P=0.02, respectively, Mann-Whitney test). There was no difference in the case of $CD8^+$ T cells (P=0.1, Mann-Whitney test, data not shown). As expected, we did not detect IFN-α- or IFN-γ-producing monocytes and mDCs (data not shown), confirming the specificity of the staining. Strikingly, in the case of pDCs, but not other cell populations, a lower relative frequency was observed in children receiving hospital-based treatment of dengue compared to children receiving non-hospital treatment (P=0.01, Mann-Whitney test, Fig. 2C), suggesting that the pDCs response is diminished in clinically severe cases of dengue. However, this was not observed when the frequencies of IFN-α-producing pDCs were compared between both treatment groups (P=0.3, non-hospital vs. hospital, Mann-Whitney test, data not shown). Similar to the inflammatory cytokines, there was no association between the type I and II IFNs *ex vivo* production and the infecting serotype (P≥0.3, Kruskal-Wallis test), the type of the infection (P≥0.5, Mann-Whitney test), the day of illness (P≥0.8, Mann-Whitney test) or the age of the patients (P≥0.4, Mann-Whitney test) (data not shown).

Table 1
Clinical and laboratory characteristics of the study cohort.

Parameters	Healthy (n=34)	Dengue			P value Healthy vs. Dengue	P value between Dengue groups
		DNS (n=15)	DWS (n=35)	SD (n=23)		
Age in months, median (range)[a]	78 (12–168)	36 (12–168)	72 (9–168)	45.5 (4–144)	0.1	0.2
Gender, male, n (%)[b]	15 (44)	8 (53.3)	11 (31.4)	10 (43.4)	0.6	0.3
Day of illness at enrollment, median (range)[a]	N/A	4 (2–7)	5 (3–7)	5 (3–8)	N/A	≤0.005[f]
Primary infection, n (%)[b]	N/A	8 (53.3)	8 (23)	7 (30)	N/A	0.1
Secondary infection, n (%)[b]	N/A	7 (46.6)	27 (77)	16 (70)	N/A	0.1
$NS1^+$, n (%)[b]	ND	12 (80)	13 (37)	14 (61)	N/A	0.01[e]
DENV-1, n (%)[b]	ND	3 (20)	0 (0)	0 (0)	N/A	0.002[f]
DENV-2, n (%)[b]	ND	2 (13.3)	6 (17)	7 (30)	N/A	0.3
DENV-3, n (%)[b]	ND	6 (40)	16 (46)	7 (30)	N/A	0.5
Undetermined, n (%)[b]	ND	4 (26.6)	13 (37)	9 (40)	N/A	0.7
Hepatomegaly, cm, median (range)[a]	0	0	2 (0–4)	5 (2–7)	< 0.0001	≤0.001[c,e]
Pleural effusion, %, median (range)[a]	0	0	0 (0–22)	19 (0–40)	< 0.0001	≤0.001[e]
Hemoglobin, g/dL, median (range)[a]	12.6 (11.2–15.7)	12 (11.1–14.5)	12.8 (11–17)	11.9 (7.7–18.9)	0.4	0.06
Hematocrit, %, median (range)[a]	37.3 (31.5–47.3)	36.7 (32.2–40.1)	38.4 (30.9–48.8)	35.6 (23.7–53.5)	0.5	0.09
White blood cells, cells/μL, median (range)[a]	8.9 (5.4–17.2)	5.74 (2.79–7.71)	4.9 (1–13.6)	8.7 (3.2–22.7)	0.005	0.002[f]
Platelets, cells x 1000/μL, median (range)[a]	322 (192–511)	109 (88–287)	82 (24–214)	59.5 (16–217)	< 0.0001	0.001[g]
AST, U/L, median (range)[a]	ND	ND	104.2 (37–192.4)	127.1 (84–2130)	N/A	0.004
ALT, U/L, median (range)[a]	ND	ND	79.05 (37.8–123.2)	81.3 (37–688)	N/A	0.02
PT, seconds, median (range)[a]	ND	ND	11.6 (10.9–14.1)	12.3 (11–100)	N/A	0.1
PTT, seconds, median (range)[a]	ND	ND	35.6 (27.2–51.2)	36.2 (29.1–100)	N/A	0.4

[a] Mann-Whitney test or Kruskal-Wallis test with Dunn's post hoc test.
[b] Fisher test or Chi square. N/A: Does not apply. ND: Not determined.
[c] DNS vs. DWS and SD.
[d] DNS vs. DWS.
[e] SD vs. DNS and DWS.
[f] DWS vs. SD.
[g] DNS vs. SD. AST: aspartate transaminase; ALT: Alanine transaminase. PT: Prothrombine time. PTT: Partial thromboplastine time.

3.4. IFNs levels in plasma are inversely associated with dengue clinical severity

Dengue virus selectively blocks the production and signaling of type I IFNs; here we demonstrated an inhibition of the production of not only IFN-α by pDCs but also IFN-γ by $CD4^+$ T cells after *ex vivo* stimulation in children with dengue. Thus, we hypothesized that this effect could be reflected in the plasma levels of the respective cytokines. As shown in Fig. 3, the plasma levels of IFN-α and IFN-γ were significantly higher in children with dengue compared to healthy controls (P≤0.009, Dunn's post hoc test), but the lowest levels in the dengue group were found in children who received a hospital-based treatment (DWS and SD) (P=0.005 and P=0.01, to IFN-α and IFN-γ levels, respectively; Dunn's post hoc test). Therefore, the levels of both cytokines were inversely associated with dengue clinical severity. According with the early production of type I IFN during viral infection, when the day of illness was compared, the IFN-α levels were higher in children in the third day of illness than in those in the seventh day (median pg/mL [range] 62.9 [5.6–1521] vs. 0.7 [0.7–32], respectively, P=0.01, Dunn's post hoc test), while there were no differences in the case of IFN-γ (P≥0.4, Kruskal-Wallis test, data not shown). When the whole panel of cytokines in plasma was analyzed, IL-6 was positively associated with clinical severity of dengue (median pg/mL [range] of 5.7 [1.5–45.3] vs. 8.9 [1.5–85.7], in non-hospital vs. hospital-based treatment, P=0.01, Dunn's post hoc test), while the levels of IL-10, TNF-α and other cytokines, such as IL-2 and IL-4, were comparable among both groups (P≥0.1, Mann-Whitney test, data not shown). In summary, the type I and II IFNs levels in plasma were higher in mild form of infections, supporting its protective role during DENV in children.

3.5. DENV modulates the production of IFN-α

The previous results support the hypothesis that DENV affects the production of cytokines by the major PBMC populations, but the IFN axis is most affected, probably through the direct blockage of recognition receptors or their signaling proteins by viral components or the induction of immune-suppressive factors (Malavige et al., 2013). To establish a mechanism of inhibition (direct or indirect viral effect) of DENV on the production of IFNs, we compared the levels of IFN-α and IFN-γ in plasma and the frequencies of IFN-α-producing pDCs and IFN-γ-producing $CD4^+$ T cells after *ex vivo* stimulation in patients with detectable and undetectable circulating virus ($NS1^+$ and/or $Viremia^+$ vs. $NS1^-$ and $Viremia^-$). As shown in Fig. 4A, children with detectable circulating virus had higher levels of IFN-α in plasma (median pg/mL [range] of 8.6 [0.7–468] vs. 1.7 [0.7–1521], $NS1^+$ and/or $Viremia^+$ vs. $NS1^-$ and $Viremia^-$, P=0.03, Mann-Whitney test), suggesting that the presence of the virus or any of its components is necessary for type I IFNs secretion. However, they had lower frequencies of IFN-α-producing pDCs (median % [range] of 0.0 [0.0–6.5] vs. 0.7 [0.0–15.2], $NS1^+$ and/or $Viremia^+$ vs. $NS1^-$ and $Viremia^-$, P=0.004, Mann-Whitney test, data not shown), and this was reflected in an inverse moderate correlation between both parameters (rho=−0.41, P=0.005, Spearman test; Fig. 4B). The previous results were not due to low relative frequencies of pDCs, as there was not correlation between the plasma levels of IFN-α and the percentage of pDCs (after 12 h of incubation without any stimulus) (rho=0.2, P=0.1; Fig. 4C). We did not find any differences or correlations among the IFN-γ plasma levels and the percentage of IFN-γ-producing $CD4^+$ T cells or their total relative frequencies (P≥0.3, Mann-Whitney and Spearman test; data not

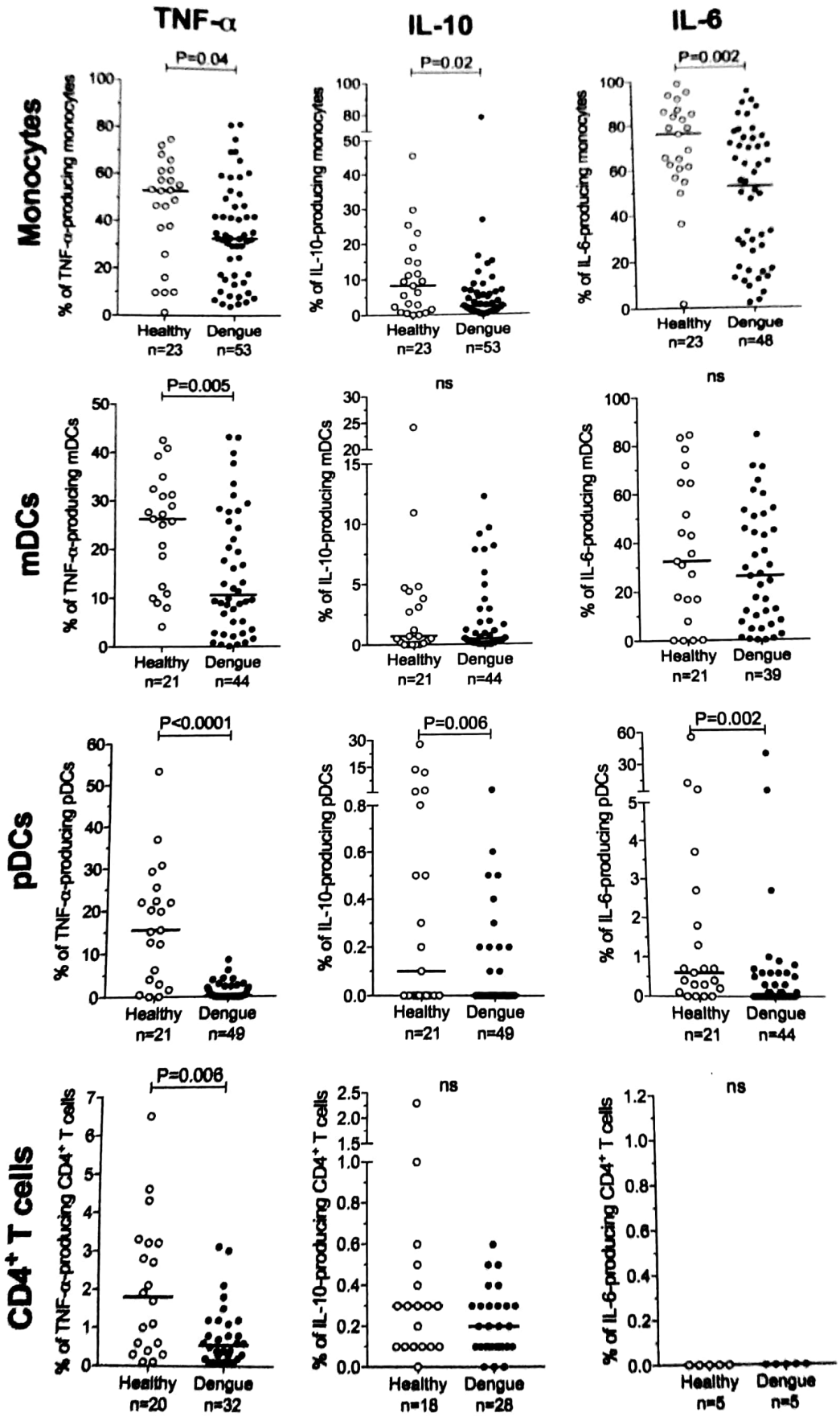

Fig. 1. DENV affects the *ex vivo* production of inflammatory cytokines in most of the PBMCs subsets. The frequencies of TNF-α, IL-10 and IL-6-producing monocytes, mDCs, pDCs and CD4+ T cells in children with dengue and healthy controls were determined through flow cytometry (analyzed as shown in Supplementary Fig. 1). The continuous lines indicate the median in each group. The P values of the Mann-Whitney test are shown. The number of individuals analyzed is shown below each group.

Table 2
Relative frequencies of PBMCs subsets.

Population (% of total PBMCs)	Healthy (n=23)	Dengue (n=52)	Mann-Whitney P value
Monocytes (CD14$^+$ cells)	5.3 (1–25.4)	3.65 (0.1–25)	0.225
B cells (CD19$^+$ cells)	5 (1.8–9.8)	3.25 (1–16.5)	0.05
CD4$^+$ T cells (CD3$^+$CD4$^+$ cells)	24.8 (5.45–40.8)	31.7 (14.99–51.9)	0.03
CD8$^+$ T cells (CD3$^+$CD8$^+$ cells)	25.3 (15–36.5)	27.2 (13.3–49.2)	0.5
mDCs (HLA-DR^{High}CD11c$^+$ cells)	0.06 (0.007–3)	0.26 (0.004–10.6)	0.02
pDCs (HLA-DR$^+$CD123$^+$ cells)	0.13 (0.02–0.4)	0.25 (0.015–1.8)	0.005

The median (range) of each cell population after 12 h of incubation without any stimulus is shown. The day at which the relative frequencies of PBMCs subsets were established is the same of the enrollment to the study (as shown in Table 1).

shown). Thus, DENV modulates the IFN-α production: it stimulates its systemic production but inhibits pDCs function.

4. Discussion

We analyzed the functionality (cytokine production after *ex vivo* stimulation) of the major PBMC subpopulations in children with dengue, finding that during the acute infection: i. monocytes, mDCs, pDCs and CD4$^+$ T cells have limited capacity to produce inflammatory cytokines; ii. pDCs and CD4$^+$ T cells also exhibit significant low production of type I and II IFNs, respectively; iii. the plasma levels of IFN-α and IFN-γ are inversely associated with clinical severity; and iv. DENV induces the systemic production of IFN-α but affects the function of pDCs.

To reveal the DENV immune subversion during natural infection, we measured the cytokine response of the major PBMC subsets after *ex vivo* stimulation with well-characterized stimuli and detected its respective levels in plasma. In agreement with previous reports (Suhart et al., 2003), in comparison with healthy controls, we found lower frequencies of TNF-α, IL-10 and IL-6-producing monocytes, mDCs and pDCs in children with dengue (Fig. 1). CD4$^+$ T cells also had lower expression of TNF-α (Fig. 1). Monocytes and pDCs were the most affected populations, while the inhibition in the production of TNF-α was generalized in all the populations evaluated, consistent possibly with the reported blockage of nuclear factor-kappa B, its main transcription factor (Chang et al., 2012). On the other hand, B cells and CD8$^+$ T cells had similar production of inflammatory cytokines compared to healthy controls after *ex vivo* stimulation, indicating that the production of the cytokines evaluated here by these cells is not affected during DENV natural infection and the DENV modulatory effect is cellular lineage-specific. As we did not find any association between the frequencies of cytokine-producing cells and the presence of any viral component, we propose that the mechanism of dysfunction of these cells could be indirect, including an exhaustive state (Chunhakan et al., 2015) secondary to a early massive immune activation (van de Weg et al., 2013), the inhibitory effect of IL-10, a cytokine highly released in DENV infection (Malavige et al., 2013) or the modulation in the expression of pattern recognition receptors (Torres et al., 2013). In the case of CD4$^+$ T cells, the mechanism is T cell receptor-independent, as we found similar inhibition results after stimulation with phorbol 12-myristate 13-acetate (an activator of the protein kinase C) and ionomicyn (a calcium ionofore) (Perdomo-Celis et al., 2016a). Receptors such as the programmed cell death (PD)-1 receptor and its ligands PD-L1 and PD-L2 that inhibit T cell proliferation and cytokine production, or the TNF-related apoptosis inducing ligand (TRAIL) may also play a role in the selective inhibition of CD4$^+$ but not CD8$^+$ T cells (Perdomo-Celis et al., 2016a; de Alwis et al., 2016). Of note, we cannot exclude the possibility of the direct effect of DENV in the low response of these populations, as the expression of PD-L1 and PD-L2 is induced in DENV-infected and bystander DCs, which could diminish their ability of cytokine production (Nightingale et al., 2008).

DENV selectively blocks the production of type I IFNs evading the recognition of immune sensors inside the cell and cleaving some intracellular proteins, mainly in the retinoic acid-inducible gene I (RIG-I) pathway. The NS3 protein prevents the translocation of RIG-I to the mitochondrial antiviral-signaling (MAVS) mediated by the 14-3-3ε protein (Chan and Gack, 2016), the NS4A protein directly binds to MAVS and prevents its interaction with RIG-I (He et al., 2016) and DENV subgenomic RNAs prevent the ubiquitination and activation of RIG-I by tripartite motif-containing protein 25 (Manokaran et al., 2015). Likewise, the DENV NS2B/3 protease cleaves the stimulator of IFN genes (Aguirre et al., 2012). Furthermore, the downstream signaling proteins IκB kinase ε and TANK-binding kinase are inhibited by NS2B/3 and NS4A, respectively, with the last one only inhibited by DENV-1 (Angleró-Rodríguez et al., 2014; Dalrymple et al., 2015). Additionally, the low levels of IFNs produced do not have a significant effect due to the blockage of the STAT proteins, critical components of

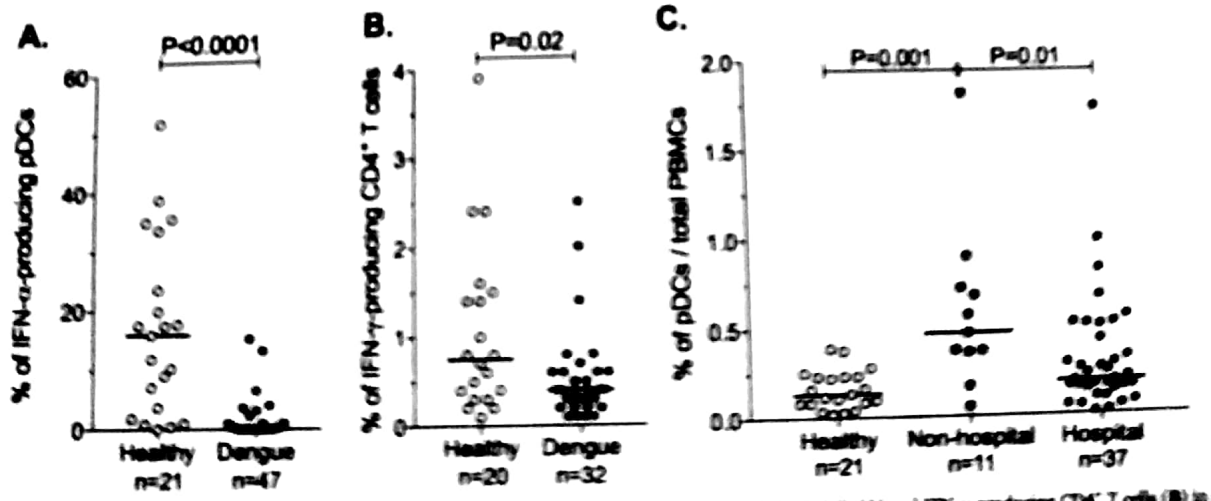

Fig. 2. DENV affects the *ex vivo* production of type I and II Interferons. The frequencies of IFN-α-producing pDCs (A) and IFN-γ-producing CD4$^+$ T cells (B) in children with dengue and healthy controls were determined through flow cytometry (analyzed as in Supplementary Fig. 1) after treatment with optimized doses of CpG 2216 or SEB, respectively. The continuous lines indicate the median in each group. The P values of the Mann-Whitney test are shown. C. Percentage of pDCs (HLA-DR$^+$Lin-1^{neg}CD123$^+$) (after 12 h of incubation without any stimulus) from total PBMCs in healthy children and children with dengue who received a non-hospital- or hospital-based treatment. The P value of Dunn's post hoc test is shown. The number of individuals analyzed is shown below each group.

children with SD but not in patients with non-severe dengue. This complication during acute dengue infection could be related to the immune evasion mechanisms developed by DENV, but until now the association has been unclear. Concurrent bacterial infection and detectable levels of circulating endotoxins have been also reported in patients acutely infected with DENV (van de Weg et al., 2013). Although, children with SD are treated in a intensive care unit (a place frequently associated with increase in the frequency of acquired bacterial infections), we hypothesize that the relevant frequency of hospital-acquired infections in patients with SD but not mild dengue could be partially associated with the diminished IFN response, as these cytokines promote the antigen presentation, induce the production of antiviral proteins and activate antimicrobial effector functions such as the production of reactive oxygen species or phagocytosis by macrophages, among others (Schneider et al., 2014; Schroder et al., 2004). In the context of microbial translocation during DENV infection (van de Weg et al., 2013), this transitory immune suppression and its relation with clinical outcome deserves to be explored in larger cohort studies.

Interestingly, children with detectable circulating virus had the highest levels of IFN-α in plasma but the lowest frequencies of IFN-α-producing pDCs after *ex vivo* stimulation (Fig. 4), indicating that DENV induces the systemic production of this cytokine but somehow is inhibiting the main cellular source of IFN-α. DENV inhibits the production of type I IFNs and T cell priming in primary human monocyte-derived DCs (Rodriguez-Madoz et al., 2010), but, in the case of pDCs, it appears to be independent of the blockage of the IFN pathway, as the virus does not actively replicate in them (Gandini et al., 2013; Sun et al., 2009), and pDCs produce IFN-α after endocytosis of the virus (Sun et al., 2009). Another explanation is the differential expression of Toll-like receptor 9 in pDCs, which could account for the low response to CpG (Torres et al., 2013), the induction of apoptosis and elimination of the activated pDCs (Martins Sde et al., 2012) or a suppressive effect of IL-10 on IFN-stimulated genes, such as those in the IFN production pathway (Ito et al., 1999). The question of the pDCs mechanism of IFNs production and inhibition during dengue infection in children remains unanswered. In this regard, our results and previous reports (Palmer et al., 2005) support the hypothesis that DENV-exposed cells have limited production of type I IFNs (and other inflammatory cytokines) and uninfected cells are responsible of the increase in the circulating levels. The fraction of infected or virus-bearing pDCs that are not eliminated by apoptosis could probably stimulate the uninfected cell types by the paracrine secretion of type I IFNs and together contribute to the IFN antiviral response. Certainly, DENV induces an IFN response but modulates it during the infection and this dynamic is associated with clinical outcome and possibly with the increased risk of concomitant bacterial infections.

5. Conclusions

DENV affects the functionality of the major PBMC subsets probably through indirect mechanisms; this effect is evident after *ex vivo* stimulation. The IFN response is highly modulated by DENV and is associated with dengue clinical severity. The dysfunction of PBMCs in dengue could play a role in clinical outcome and the appearance of complications such as nosocomial infections in DENV infected children.

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Competing interests

The authors have no competing interests to disclose

Author contributions

CFN designed the study. FP-C did the experiments. FP-C and CFN analyzed and interpreted the data and wrote the manuscript. DMS helped in the patients enrolling and the acquisition of the clinical data. All authors gave final approval of the version to be submitted.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi 10.1016/j.virol.2017.04.004.

References

Aguirre, S., Maestre, A.M., Pagni, S., Patel, J.R., Savage, T., Gutman, D., et al., 2012. DENV inhibits type I IFN production in infected cells by cleaving human STING. PLoS Pathog. 8, e1002934. http://dx.doi.org/10.1371/journal.ppat.1002934.

de Alwis, R., Bangs, D.J., Angelo, M.A., Cerpas, C., Fernando, A., Sidney, J., et al., 2016. Immunodominant dengue virus-specific CD8+ T cell responses are associated with a memory PD-1+ phenotype. J. Virol. 90, 4771–4779. http://dx.doi.org/10.1128/JVI.02992-15.

Angleró-Rodríguez, Y.I., Pantoja, P., Sariol, C.A., 2014. Dengue virus subverts the interferon induction pathway via NS2B/3 protease-IkappaB kinase epsilon interaction. Clin. Vaccin. Immunol.: CVI 21, 29–38. http://dx.doi.org/10.1128/cvi.00000-13.

Ashour, J., Laurent-Rolle, M., Shi, P.Y., García-Sastre, A., 2009. NS5 of dengue virus mediates STAT2 binding and degradation. J. Virol. 83, 5408–5418. http://dx.doi.org/10.1128/JVI.02188-08.

Bhatt, S., Gething, P.W., Brady, O.J., Messina, J.P., Farlow, A.W., Moyes, C.L., et al., 2013. The global distribution and burden of dengue. Nature 496, 504–507. http://dx.doi.org/10.1038/nature12060.

Bueno, C., Almeida, J., Alguero, M.C., Sanchez, M.L., Vaquero, J.M., Laso, F.J., et al., 2001. Flow cytometric analysis of cytokine production by normal human peripheral blood dendritic cells and monocytes: comparative analysis of different stimuli, secretion-blocking agents and incubation periods. Cytometry 46, 33–40.

Chan, Y.K., Gack, M.U., 2016. A phosphomimetic-based mechanism of dengue virus to antagonize innate immunity. Nat. Immunol. 17, 523–530. http://dx.doi.org/10.1038/ni.3393.

Chang, T.H., Chen, S.R., Yu, C.Y., Lin, Y.S., Chen, Y.S., Kubota, T., et al., 2012. Dengue virus serotype 2 blocks extracellular signal-regulated kinase and nuclear factor-kappaB activation to downregulate cytokine production. PloS One 7, e41635. http://dx.doi.org/10.1371/journal.pone.0041635.

Chen, R.F., Yang, K.D., Wang, L., Liu, J.W., Chiu, C.C., Cheng, J.T., 2007. Different clinical and laboratory manifestations between dengue haemorrhagic fever and dengue fever with bleeding tendency. Trans. R. Soc. Trop. Med Hyg. 101, 1106–1113. http://dx.doi.org/10.1016/j.trstmh.2007.06.019.

Chunhakan, S., Butthep, P., Yoksan, S., Tangnararatchakit, K., Chuansumrit, A., 2015. Vascular leakage in dengue hemorrhagic Fever is associated with dengue infected monocytes, monocyte activation/exhaustion, and cytokines production. Int. J. Vasc. Med. 2015, 917143. http://dx.doi.org/10.1155/2015/917143.

Colonna, M., Trinchieri, G., Liu, Y.J., 2004. Plasmacytoid dendritic cells in immunity. Nat. Immunol. 5, 1219–1226. http://dx.doi.org/10.1038/ni1141.

Dalrymple, N.A., Cimica, V., Mackow, E.R., 2015. dengue virus NS proteins inhibit RIG-I/MAVS signaling by blocking TBK1/IRF3 phosphorylation: dengue virus serotype 1 NS4A Is a unique interferon-regulating virulence determinant. mBio 6, e00553–15. http://dx.doi.org/10.1128/mBio.00553-15.

De Carvalho Bittencourt M, Martial, J., Cabie, A., Thomas, L., Cesaire, R., 2012. Decreased peripheral dendritic cell numbers in dengue virus infection. J. Clin. Immunol. 32, 161–172. http://dx.doi.org/10.1007/s10875-011-9592-9.

De La Cruz Hernandez, S.I., Puerta-Guardo, H., Flores-Aguilar, H., Gonzalez-Mateos, S., Lopez-Martinez, I., Ortiz-Navarrete, V., et al., 2014. A strong interferon response correlates with a milder dengue clinical condition. J. Clin. Virol.: Off. Publ. Pan Am. Soc. Clin. Virol. 60, 196–199. http://dx.doi.org/10.1016/j.jcv.2014.04.002.

Dengue, 2009. Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control: New Edition. Geneva.

Gack, M.U., Diamond, M.S., 2016. Innate immune escape by Dengue and West Nile viruses. Curr. Opin. Virol. 20, 119–128. http://dx.doi.org/10.1016/

j.coviro.2016.09.013.
Gandini, M., Gras, C., Azeredo, E.L., Pinto, L.M., Smith, N., Despres, P., et al., 2013. Dengue virus activates membrane TRAIL relocalization and IFN-alpha production by human plasmacytoid dendritic cells in vitro and in vivo. PLoS Negl. Trop. Dis. 7, e2257. http://dx.doi.org/10.1371/journal.pntd.0002257.
Green, A.M., Beatty, P.R., Hadjilaou, A., Harris, E., 2014. Innate immunity to dengue virus infection and subversion of antiviral responses. J. Mol. Biol. 426, 1148–1160. http://dx.doi.org/10.1016/j.jmb.2013.11.023.
He, Z., Zhu, X., Wen, W., Yuan, J., Hu, Y., Chen, J., et al., 2016. Dengue virus subverts host innate immunity by targeting adaptor protein MAVS. J. Virol. 90, 7219–7230. http://dx.doi.org/10.1128/jvi.00221-16.
Ito, S., Ansari, P., Sakatsume, M., Dickensheets, H., Vazquez, N., Donnelly, R.P., et al., 1999. Interleukin-10 inhibits expression of both interferon alpha- and interferon gamma- induced genes by suppressing tyrosine phosphorylation of STAT1. Blood 93, 1456–1463.
Kurane, I., Innis, B.L., Nimmannitya, S., Nisalak, A., Meager, A., Ennis, F.A., 1993. High levels of interferon alpha in the sera of children with dengue virus infection. Am. J. Trop. Med. Hyg. 48, 222–229.
Lanciotti, R.S., Calisher, C.H., Gubler, D.J., Chang, G.J., Vorndam, A.V., 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J. Clin. Microbiol. 30, 545–551.
Malavige, G.N., Jeewandara, C., Alles, K.M., Salimi, M., Gomes, L., Kamaladasa, A., et al., 2013. Suppression of virus specific immune responses by IL-10 in acute dengue infection. PLoS Negl. Trop. Dis. 7, e2409. http://dx.doi.org/10.1371/journal.pntd.0002409.
Manokaran, G., Finol, E., Wang, C., Gunaratne, J., Bahl, J., Ong, E.Z., et al., 2015. Dengue subgenomic RNA binds TRIM25 to inhibit interferon expression for epidemiological fitness. Science 350, 217–221. http://dx.doi.org/10.1126/science.aab3369.
Martins Sde, T., Silveira, G.F., Alves, L.R., Duarte dos Santos, C.N., Bordignon, J., 2012. Dendritic cell apoptosis and the pathogenesis of dengue. Viruses 4, 2736–2753. http://dx.doi.org/10.3390/v4112736.
Mathew, A., Kurane, I., Green, S., Vaughn, D.W., Kalayanarooj, S., Suntayakorn, S., et al., 1999. Impaired T cell proliferation in acute dengue infection. J. Immunol. 162, 5609–5615.
Ministerio de Salud y Protección Social, 2015. Casos clínicos y memorias del curso de enfermedades transmitidas por vectores Dengue y Chikunguña.
Morrison, J., Aguirre, S., Fernandez-Sesma, A., 2012. Innate immunity evasion by Dengue virus. Viruses 4, 397–413. http://dx.doi.org/10.3390/v4030397.
Munoz-Jordan, J.L., Laurent-Rolle, M., Ashour, J., Martinez-Sobrido, L., Ashok, M., Lipkin, W.I., et al., 2005. Inhibition of alpha/beta interferon signaling by the NS4B protein of flaviviruses. J. Virol. 79, 8004–8013. http://dx.doi.org/10.1128/JVI.79.13.8004-8013.2005.
Nightingale, Z.D., Patkar, C., Rothman, A.L., 2008. Viral replication and paracrine effects result in distinct, functional responses of dendritic cells following infection with dengue 2 virus. J. Leukoc. Biol. 84, 1028–1038. http://dx.doi.org/10.1189/jlb.0208105.
Olagnier, D., Amatore, D., Castiello, L., Ferrari, M., Palermo, E., Diamond, M.S., et al., 2016. Dengue Virus Immunopathogenesis: lessons applicable to the emergence of Zika Virus. J. Mol. Biol. 428, 3429–3448. http://dx.doi.org/10.1016/j.jmb.2016.04.024.
Palmer, D.R., Sun, P., Celluzzi, C., Bisbing, J., Pang, S., Sun, W., et al., 2005. Differential effects of dengue virus on infected and bystander dendritic cells. J. Virol. 79, 2432–2439. http://dx.doi.org/10.1128/JVI.79.4.2432-2439.2005.
Perdomo-Celis, F., Salgado, D.M., Castaneda, D.M., Narvaez, C.F., 2016a. Viability and functionality of cryopreserved peripheral blood mononuclear cells in pediatric dengue. Clin. Vaccin. Immunol.: Cvi. 23, 417–426. http://dx.doi.org/10.1128/CVI.00038-16.
Perdomo-Celis, F., Salgado, D.M., Narvaez, C.F., 2016b. Magnitude of viremia, antigenemia and infection of circulating monocytes in children with mild and severe dengue. Acta Trop. 167, 1–8. http://dx.doi.org/10.1016/j.actatropica.2016.12.011.
Pichyangkul, S., Endy, T.P., Kalayanarooj, S., Nisalak, A., Yongvanitchit, K., Green, S., et al., 2003. A blunted blood plasmacytoid dendritic cell response to an acute systemic viral infection is associated with increased disease severity. J. Immunol. 171, 5571–5578.
Rodriguez-Madoz, J.R., Bernal-Rubio, D., Kaminski, D., Boyd, K., Fernandez-Sesma, A., 2010. Dengue virus inhibits the production of type I interferon in primary human dendritic cells. J. Virol. 84, 4845–4850. http://dx.doi.org/10.1128/JVI.02514-09.
Rothman, A.L., 2011. Immunity to dengue virus: a tale of original antigenic sin and tropical cytokine storms. Nat. Rev. Immunol. 11, 532–543. http://dx.doi.org/10.1038/nri3014.
Schneider, W.M., Chevillotte, M.D., Rice, C.M., 2014. Interferon-stimulated genes: a complex web of host defenses. Annu. Rev. Immunol. 32, 513–545. http://dx.doi.org/10.1146/annurev-immunol-032713-120231.
Schroder, K., Hertzog, P.J., Ravasi, T., Hume, D.A., 2004. Interferon-gamma: an overview of signals, mechanisms and functions. J. Leukoc. Biol. 75, 163–189. http://dx.doi.org/10.1189/jlb.0603252.
See, K.C., Phua, J., Yip, H.S., Yeo, L.L., Lim, T.K., 2013. Identification of concurrent bacterial infection in adult patients with dengue. Am. J. Trop. Med. Hyg. 89, 804. http://dx.doi.org/10.4269/ajtmh.13-0197.
Shresta, S., Kyle, J.L., Snider, H.M., Basavapatna, M., Beatty, P.R., Harris, E., 2004. Interferon-dependent immunity is essential for resistance to primary dengue virus infection in mice, whereas T- and B-cell-dependent immunity are less critical. J. Virol. 78, 2701–2710.
Suharti, C., van Gorp, E.C., Dolmans, W.M., Setiati, T.E., Hack, C.E., Djokomoeljanto, R., et al., 2003. Cytokine patterns during dengue shock syndrome. Eur. Cytokine Netw. 14, 172–177.
Sun, P., Fernandez, S., Marovich, M.A., Palmer, D.R., Celluzzi, C.M., Boonnak, K., et al., 2009. Functional characterization of ex vivo blood myeloid and plasmacytoid dendritic cells after infection with dengue virus. Virology 383, 207–215. http://dx.doi.org/10.1016/j.virol.2008.10.022.
Toro, J.F., Salgado, D.M., Vega, R., Rodriguez, J.A., Rodriguez, L.S., Angel, J., et al., 2016. Total and envelope protein-specific antibody-secreting cell response in pediatric dengue is highly modulated by age and subsequent infections. PloS One 11, e0161795. http://dx.doi.org/10.1371/journal.pone.0161795.
Torres, S., Hernandez, J.C., Giraldo, D., Arboleda, M., Rojas, M., Smit, J.M., et al., 2013. Differential expression of Toll-like receptors in dendritic cells of patients with dengue during early and late acute phases of the disease. PLoS Negl. Trop. Dis. 7, e2060. http://dx.doi.org/10.1371/journal.pntd.0002060.
Ubol, S., Phuklia, W., Kalayanarooj, S., Modhiran, N., 2010. Mechanisms of immune evasion induced by a complex of dengue virus and preexisting enhancing antibodies. J. Infect. Dis. 201, 923–935. http://dx.doi.org/10.1086/651018.
van de Weg, C.A., Koraka, P., van Gorp, E.C., Mairuhu, A.T., Supriatna, M., Soemantri, A., et al., 2012. Lipopolysaccharide levels are elevated in dengue virus infected patients and correlate with disease severity. J. Clin. Virol.: Off. Publ. Pan Am. Soc. Clin. Virol. 53, 38–42. http://dx.doi.org/10.1016/j.jcv.2011.09.028.
van de Weg, C.A., Pannuti, C.S., de Araujo, E.S., van den Ham, H.J., Andeweg, A.C., Boas, L.S., et al., 2013. Microbial translocation is associated with extensive immune activation in dengue virus infected patients with severe disease. PLoS Negl. Trop. Dis. 7, e2236. http://dx.doi.org/10.1371/journal.pntd.0002236.
Villiger, P.M., Cronin, M.T., Amenomori, T., Wachsman, W., Lotz, M., 1991. IL-6 production by human T lymphocytes. expression in HTLV-1-infected but not in normal T cells. J. Immunol. 146, 550–559.