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T helper type 2 bias and type 17 suppression in primary dengue virus infection in infants and young children

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Background: The immune response to dengue virus (DENV) primary infection in infants and young children is not well characterized. In Northern Argentina, >90% of the population was DENV-naïve before the 2009 outbreak, allowing evaluation of age-dependent primary responses to infection.

Methods: We conducted a comparative study of the immune response to DENV in 27 infected infants, young children and their mothers. Lymphocyte T helper (Th) 1, Th2, Th17 and inflammatory responses were assayed in blood during the 2009 DENV-1 epidemic.

Results: The immune response to DENV-1 was significantly biased to Th2 in infected infants and young children, compared to infants with other febrile illnesses (for IL-4 $p < 0.001$) and to their infected mothers (for IL-4 $p < 0.01$). In addition, IL-17 suppression was observed in the memory response to DENV-1 in infected infants ($p < 0.01$ vs placebo).

Conclusion: Age-related differences in the primary response to DENV, characterized by an immature Th2 polarization and Th17 suppression in infants, should be studied further in order to expand our understanding of the mechanism of dengue pathogenesis.

Keywords: Dengue virus, Infant, Immune immaturity, Th2 polarization

Introduction

The four serotypes of dengue virus (DENV 1–4) cause the most prevalent arthropod-borne disease in humans, with an estimated one hundred million cases of a self-limited febrile illness characterized by myalgias and rash every year.[1] In approximately 500 000 patients who are infected with DENV annually, infection results in severe disease characterized by thrombocytopenia, capillary leakage, bleeding and transaminitis, and may progress to hypovolemic shock.[2,3] Approximately 5% of cases of severe dengue (formerly known as dengue hemorrhagic fever [DHF]/dengue shock syndrome [DSS]) are observed in infants, who are thought to experience antibody dependent enhancement (ADE) of infection as a consequence of transplacentally-acquired sub-neutralizing levels of maternal antibody.[4–6] Interestingly, the primary immune response of infants to DENV is not well characterized.

In Northern Argentina, 25 000 cases of dengue were reported in 2009.[7] And since <10% of women of childbearing age had been previously infected with DENV,[7] most susceptible infants and their mothers exposed to the virus experienced primary DENV infections while lacking antibody to modulate the disease. Primary infection often occurred simultaneously in infants/young children and their biological mothers, providing a unique opportunity to discriminate potentially important aspects of age-dependent immune responses to DENV.

The immune system of young infants is immature, making them susceptible to severe illness from many infectious diseases that are uncommon at later ages.[8,9] Typical manifestations of immune immaturity in other conditions include a type 2 bias of the $CD4^+$ T helper (Th) response and deficits in inflammation enhancing susceptibility to infections.[10] In this context, and given the importance of understanding the characteristics of the immune response to dengue in infants, we compared the adaptive Th lymphocyte

response associated with type 1 vs type 2 polarization (interferon-γ [IFN-γ] vs interleukin-4 [IL-4] production) and inflammation in DENV-infected infants/young children and their mothers during the 2009 epidemic, in a region with low previous prevalence of infections due to the virus.

Materials and methods

Study populations

Data were collected during a DENV-1 epidemic in March 2009 affecting the city of Oran in the province of Salta in Argentina (Figure 1). Children under the age of 24 months and their mothers with symptoms of probable dengue upon presentation were invited to participate in the study. Demographic and clinical information were obtained, and admitted children were followed as inpatients in the local hospital until symptoms resolved. Between 21 and 28 days after initial presentation, participating families were visited at home and 1–2 ml of blood obtained from formerly symptomatic mothers and children.[11,12] In the case of one child with severe congenital dengue virus infection and prolonged hospitalization, blood was obtained from the DENV-1 infected infant while hospitalized (his mother had been discharged).

In the winter and spring before the epidemic, we conducted a serosurvey to assess seropositivity for DENV serotypes in women seeking obstetric care in the region; 310 pregnant women participated in the study. Recruitment was conducted while women were hospitalized for delivery. Blood was drawn (2 ml), serum obtained after 15 min centrifugation and stored at -20°C.

Figure 1. Geographical location of the city of Oran in the province of Salta, Argentina.

Identification of DENV-1 positive patients

Blood was obtained from all patients during acute illness and DENV positive cases were confirmed by reverse transcription-polymerase chain reaction (RT-PCR) amplification of viral RNA[13] and/or the presence of IgM and/or IgG by ELISA (Focus Technologies Cypress, CA, USA) in paired acute and convalescent-phase serum samples. Primary and secondary cases were differentiated based on serology.[14] Patients were then divided in two groups: a group of DENV-1 positive patients and a group found to have other febrile illnesses (OFI) with similar clinical manifestations.

Description of clinical manifestations

The following definitions were used to evaluate each of the signs and symptoms in infants: fever: a temperature ≥38°C; myalgia: elicited by gentle pressure during physical examination; petechiae: a purpuric skin lesion <3 mm in diameter reflecting a minor hemorrhage; vomiting: defined as more than three episodes in one day; diarrhea: three or more loose or liquid stools per day; abdominal pain: abdominal tenderness or continuous pain in physical examination; pruritis: evidence of an unpleasant cutaneous sensation with highly variable intensity; lethargy: state of drowsiness or deep stupor. Morbidity was defined as a disease state of an individual having any of the previously described signs and symptoms.

Purification of DENV E protein ectodomains

DENV-1 E (FGA/89) and DENV-2 E (FGA/2002) cloned into pMT-Bip (Invitrogen, Carlsbad, CA, USA) were used for co-transfection of S2 cells. Stable cell lines were obtained after 4 weeks of selection with 10 μg/ml Blasticidin (Invitrogen) and recombinant protein expression was induced with 500 μM $CuSO_4$ for 7–9 days. A metal-stripped chelating sepharose column (GE Healthcare, Little Chalfont, Buckinghamshire, UK) was used for purification of DENV-1 E and a nickel-sepharose HP column (GE Healthcare, UK) for DENV-2 E. DENV-3 E (paH881, Thailand) and DENV-4 E (63632, Myanmar) were generated as previously described.[15,16]

Isolation and stimulation of peripheral blood mononuclear cells (PBMC)

Blood (from day 21–28 after onset of illness) was obtained in tubes containing EDTA. PBMC from dengue and OFI groups were isolated using Ficoll-Plaque Plus (GE Healthcare, Uppsala, Sweden) and $5x10^5$ cells were incubated per well in 200 μl of RPMI 1640 medium (GIBCO, Grand Island, NY, USA) with DENV-1 envelope (E) protein (E-DENV-1) (1 μg/200 μl) for IFN-γ and IL-4 determinations in all subjects and IL-17A in children, UV-irradiated DENV-1 (UV-DENV-1) (MOI equivalent of 1) for all other assays, or RPMI medium alone (placebo). Placebo recipients were the same patients from the dengue and OFI groups but, in this case, cells were incubated with RPMI only (no virus). Since no difference in cytokine levels was observed after placebo stimulation of PBMC between those infected with DENV during the outbreak (dengue group) and those infected with other agents (OFI group), both

groups were analyzed together in one group of patients providing cells after convalescence and without antigen specific stimulation (placebo group). In addition, PBMC from three infants with congenital dengue were incubated with E proteins from serotypes 1 to 4 at 1 μg/200 μl concentration. Supernatant fluids were removed after 72 h of incubation and assayed for cytokines.

Cytokine determinations

Cytokines were determined in the supernatants of stimulated PBMCs using immunoassays for IL-6 (BD Biosciences, San Diego, CA, USA), IL-4, IFN-γ, IL-17A, IL-23, IL-1β and TNF-α (Invitrogen) following the manufacturers' instructions. Small blood volumes obtained from convalescent infants and young children precluded testing of every sample for all cytokines.

Determination of IgG titers

Sera (from days 21–28 after acute infection) from DENV-1 infected children and their infected mothers were used to compare anti-DENV IgG titers using a commercial immunoassay (Focus Technologies), following the manufacturer's instructions. Briefly, antigen-coated wells (including equal proportions of inactivated and purified DENV serotypes 1 to 4) were washed with wash buffer solution and then incubated with each diluted sample, control or calibrator for 60 min at room temperature (20–25°C). The wells were then washed three times and peroxidase-conjugated goat anti-human IgG added to all wells. After washing the wells again, TMB substrate was added and wells were incubated for 10 minutes at room temperature (20–25°C). Finally, the reaction was stopped with 1M sulfuric acid and optical density (OD) values were measured at 450 nm. All patient results were reported as index values relative to the cut-off calibrator. In order to calculate index values, specimen OD values (corrected by blank readings) were divided by the mean of the corrected cut-off calibrator OD values. An index value of >1.00 is presumptive for the presence of IgG antibodies to DENV. An index value of <1.00 indicates no IgG antibodies to DENV were detected.

Cell culture and virus preparation

Vero (African green monkey kidney) cells were grown in MEM (GIBCO, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS). The C6/36 HT mosquito cell line from *Aedes albopictus*, adapted to grow at 33°C, was cultured in L-15 Medium (Leibovitz; GIBCO, Grand Island, NY, USA) supplemented with 0.3% tryptose phosphate broth, 0.02% glutamine, 1% MEM non-essential amino acids solution and 5% FBS. DENV-1 strain HW, DENV-3 strain H-87, and DENV-4 strain 8124 were generously provided by Dr. Elsa Damonte (Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina) and DENV-2 strain 16681 was kindly obtained from Dr. Andrea Gamarnik (Fundacion Instituto Leloir, Buenos Aires, Argentina). Virus stocks were prepared in C6/36 HT cells and titrated by plaque formation in Vero cells.

Neutralization assay

To determine anti-DENV antibody prevalence in women of childbearing age, previous DENV infections were assessed in women using plaque reduction neutralization assays against all DENV serotypes. Briefly, sera were heat-inactivated at 56°C for 30 min and four-fold serial dilutions of the serum samples (1/10 to 1/2560) in MEM were mixed with an equal volume containing 100 PFU of each DENV serotype in MEM containing 2% FBS and 10% complement sera from guinea pig. The mixtures were incubated at 37°C for 1 h and then transferred to Vero cells plated in 24-well plates. After 1 h incubation at 37°C, cells were overlaid with MEM 2% FBS containing 1% methylcellulose (Sigma, Saint Louis, MO, USA) and further incubated at 37°C for 7 days. Cells were fixed with acetone-methanol (50:50) and immunostained with a DENV E-specific mouse ascitic fluid for each DENV serotype (provided by CDC), followed by an incubation with goat anti-mouse HRP-conjugated IgG (KPL, Gaithersburg, MD, USA). Finally, DAB substrate (Sigma) was added to wells and after washing, plaques were counted. Neutralization titers were determined as the reciprocal of the highest serum dilution yielding a 50% reduction in viral plaques.

Statistical analysis

Data were analyzed using the χ^2 test for proportions and Student's t or Mann Whitney tests for continuous variables where appropriate. Statistical analyses were performed using a Stata 10.0 package for IBM-PC (Stata Corp, College Station, TX, USA). A Bonferroni correction was used to account for multiple cytokine comparisons. Because three Th cytokine pathways were explored, statistically significant implies $p < 0.0167$ to control the family-wise type I error at 5%. A p value <0.05 was considered significant for Th ratios and comparison of secondary cytokines.

Results

Study population

Fifty-four patients participated in our study; 27 infants and young children and their mothers presented symptoms compatible with probable dengue during the 2009 epidemic and were invited to join these investigations. Among the participants, 9 children (33%) and 14 mothers (52%) were confirmed to have dengue (DENV-1 confirmed by RT-PCR), while 18 children (67%) and 13 mothers (48%) had OFI.

Pediatric age distributions were similar in the dengue and OFI groups with an average age of 7.7 (range, 0–24 months) and 9.2 (range, 0–20 months) months, respectively. Two children (22%) in the dengue group and 11 (61%) in the OFI group were males. Three of the children with dengue presented symptoms at birth or during the first day of life and were diagnosed with congenital dengue. Of these congenital cases, all were born at term and required hospitalization as neonates. None of these children died as a result of dengue. One of these infants had signs and symptoms of severe dengue, including bleeding (epistaxis), persistent thrombocytopenia and transaminitis. A detailed account of his clinical and laboratory manifestations was reported elsewhere.[17] The two other infants with congenital infection and the six additional children with confirmed dengue had mild symptoms (Table 1). Two of the children grouped as OFI and three of those with dengue (the three congenital cases) required hospitalization.

In the context of the epidemic, we conducted a serosurvey of 310 pregnant women in the region and found 7.4% seropositivity for non-DENV-1 dengue viruses (i.e.: pregnant women in whom a

potential ADE-like phenomenon could be observed) and 5.5% seropositivity for DENV-1. Only 4.8% (15) and 2.6% (8) of mothers were respectively seropositive for DENV-2 and DENV-3 before the season in our serosurvey. Therefore, it is improbable that heterologous secondary infections were frequent among this group of patients. In fact, serologic assessment of the immune response to infection suggested all but one case to be primary dengue. Twenty-six mothers presented mild disease while one, delivering a baby with severe congenital illness,[17] required prolonged hospitalization herself. This was the only mother with manifestations of severe dengue and presence of IgG for DENV in acute sera. Upon resolution of symptoms, anti-DENV-1 IgG titers were assayed by ELISA and exhibited no difference between infected infants and their mothers ($p = 0.13$). For evaluation of the inflammatory profile in mothers and children with dengue, we conducted two different analyses. First, we assessed the immune response in all mothers and children participating in the study. A second analysis was performed excluding the mother with secondary infection and her child with congenital illness.

Enhanced Th2 bias in infants and young children with dengue

We first characterized the Th bias of the adaptive immune response against DENV-1 in infants and children in the study (Figure 2). Given our small sample size and the small volumes of blood that could be obtained from these young infants, we focused on three paradigmatic cytokines representing different Th biases: production of the Th1 cytokine IFN-γ, the Th2 cytokine IL-4 and the Th17 cytokine IL-17 were compared in supernatant fluids of PBMCs obtained from patients with confirmed dengue or OFI (Figure 2). IFN-γ production was higher in mononuclear cells of dengue-infected children stimulated with E-DENV1 protein compared to placebo ($p = 0.011$; because three Th cytokine pathways were explored, statistically significant implies $p < 0.0167$ to control the family-wise type I error at 5%), but similar to IFN-γ production in cells from children with OFI stimulated with E-DENV1 ($p = 0.075$; Figure 2A). Conversely, IL-4 production was enhanced ($p < 0.001$ vs OFI and $p = 0.0017$ vs placebo; Figure 2B) in cells from DENV-infected children. Comparison of Th2/Th1 ratios revealed a Th2 bias in the immune response of infants and young children infected with the virus ($p = 0.018$ vs placebo; Figure 2C). When the infant with congenital illness whose mother was suffering from secondary dengue infection was excluded from the analysis, IL-4 production remained enhanced in mononuclear cells from DENV-infected children (Figure 2B) and IFN-γ levels were similar in dengue-infected children compared to those with OFI or stimulated with placebo (Figure 2A).

Enhanced Th2 bias in responses of DENV-infected infants and children compared to their mothers

Following these observations, we then compared the Th2 responses in infants and young children to their mothers', and explored whether T helper profiles were affected by age-dependent differences (Figure 2). IL-4 levels were significantly higher in young subjects compared to adults ($p = 0.0061$;

Table 1. Demographic information and clinical manifestations of infants and young children in 2009 DENV-1 outbreak in Oran City, Salta, Argentina

	Dengue[a], n = 9		Other febrile illness[b], n = 18	
Demographic characteristics				
Mean age in months (SD)	7.7 (8.3)		9.2 (6.8)	
Male (%)	2 (22)		11 (61)	
	No. (%)	Days[c] ± SD	No. (%)	Days ± SD
Congenital/perinatal cases	3 (33)	NA	0	NA
Morbidity	9 (100)	4.7 ± 2.2	18 (100)	3.5 ± 1.9
Mortality	0	NA	0	NA
Clinical signs and symptoms				
Fever	8 (89)	4.8 ± 2.2	16 (89)	3.2 ± 1.8
Myalgia	3 (33)	5.3 ± 1.2	9 (50)	4.8 ± 2.4
Exanthema	4 (44)	2.8 ± 1.6	6 (33)	3.0 ± 1.5
Petechiae	4 (44)	2.8 ± 1.6	4 (22)	4.0 ± 1.4
Vomiting	3 (33)	4.5 ± 2.6	7 (39)	1.7 ± 0.9
Diarrhea	2 (22)	2.5 ± 1.5	7 (39)	3.8 ± 2.5
Abdominal pain	1 (11)	6.0 ± 4.2	4 (22)	4.0 ± 1.6
Pruritis	3 (33)	4.7 ± 1.3	7 (39)	3.5 ± 1.7
Lethargy	2 (22)	5.5 ± 3.2	3 (17)	2.3 ± 1.1

NA: not applicable.
[a]DENV-1 confirmed positive cases.
[b]DENV-1 negative cases (OFI).
[c]Mean duration of symptom in days.

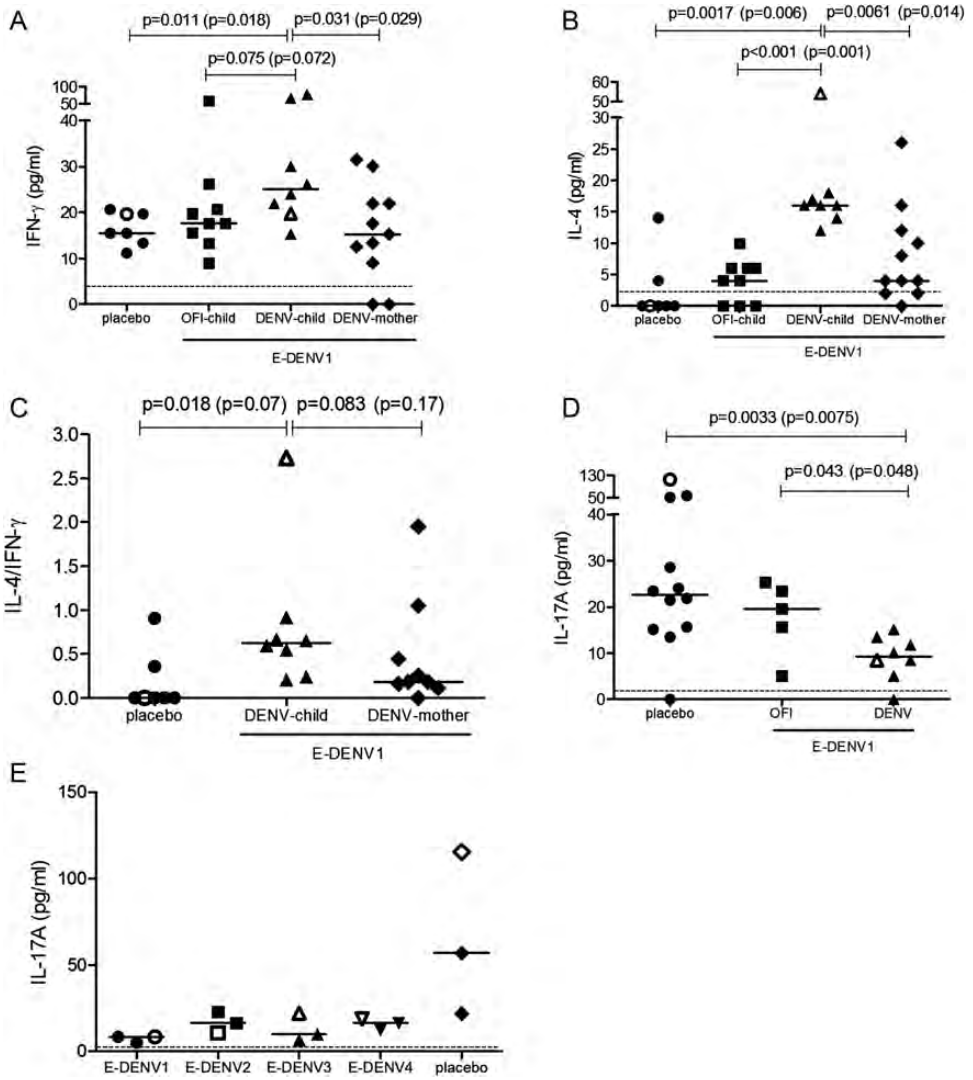

Figure 2. Th profiles after primary dengue in infants. (A – D) Cytokine levels in supernatant fluids of PBMCs from DENV-1-infected infants and young children (triangles, circles) or those presenting OFI (squares, circles), compared to those from DENV-1 infected mothers (diamonds). PBMCs were incubated with E-protein from DENV-1 (E-DENV1) or placebo. (A) IFN-γ, (B) IL-4, (C) IL-4/ IFNγ ratio and (D) IL-17A were analyzed in the supernatants. (E) IL-17A in supernatant fluids of PBMCs from three infants with congenital/perinatal DENV-1 infection incubated for 72 h with E-DENV1 (circles), E-DENV2 (squares), E-DENV3 (up-pointing triangles), E-DENV4 (down-pointing triangles) or with placebo (diamonds). Lines represent median values. Dashed lines indicate minimum detectable levels for each cytokine. For IFN-γ, IL-4, and IL-17A, the minimum detectable levels were 4 pg/ml, 2 pg/ml, and 2 pg/ml, respectively. The infant with congenital illness, whose mother had secondary DENV infection, is indicated with open figures; p values shown between parentheses correspond to data analysis excluding this infant.

Figure 2B) confirming the age-dependent bias in immune responses. Th2/Th1 ratios also exhibited a trend towards allergic responses in young infants and children compared to their mothers ($p = 0.083$; Figure 2C). Exclusion of the infant with severe congenital dengue whose mother had secondary infection, did not affect significance of IL-4 levels when comparing infants and their mothers ($p = 0.014$; Figure 2B).

Inflammation in infants and young children

IL-17A production showed suppression in E-DENV1-stimulated cells from dengue-infected children compared to cells from the same individuals exposed to placebo ($p = 0.0033$; Figure 2D). Exclusion of the infant suffering from congenital dengue did not modify the suppressive effect observed in dengue infected children. Interestingly, albeit limited by small sample size, evaluation of IL-17A production by PBMCs from the three infants with congenital disease revealed suppression of cytokine production after incubation with E proteins of the four DENV serotypes E-DENV1, E-DENV2, E-DENV3 and E-DENV4 (Figure 2E). These findings show that primary dengue in infants and young children is potentially associated with Th2 polarization and suppression of IL-17 production.

IL-1β is a pro-inflammatory cytokine released by human monocytes after activation of pattern recognition receptors (PRR) that

promote Th17 polarization from naïve T cells.[18] We therefore examined whether IL-1β levels were also affected in infants and young children with dengue (Figure 3A). IL-1β production was not significantly different in children with confirmed dengue or OFI ($p = 0.19$), and did not differ from the inflammatory cytokine response observed in cells incubated with placebo ($p = 0.85$; Figure 3A).

Another pro-inflammatory cytokine, IL-6, also promotes Th17 development; IL-6 responses were also similar in DENV-1-stimulated PBMCs of children with confirmed dengue compared to those with OFI ($p = 0.065$). Moreover, no difference in IL-23 production (a cytokine not associated with early IL-17 production, but required for expansion and maintenance of Th17 cells[19]) was observed between groups of infants and children with dengue or OFI ($p = 0.94$; Figure 3C). These findings show that, unlike the Th2 bias affecting dengue infected young children, the pro-inflammatory cytokines required for Th17 development and IL-17 production are not significantly affected in infants and young children with primary dengue.

Tumor necrosis factor-α (TNF-α) is a cytokine involved in systemic inflammation and the regulation of other immune cells.[20] It can induce fever and apoptotic cell death while also affecting viral replication. Enhanced production of TNF-α has been implicated in severe dengue and has been associated with hemorrhagic and shock-like manifestations.[20] To determine whether primary dengue in naïve infants was associated with modulation of TNF-α, we compared production of the cytokine in infected children and those with OFI (Figure 3D). Interestingly, children with confirmed primary dengue exhibited similar TNF-α production compared to children with other illnesses ($p = 0.27$). Similar results were obtained for IL-1β, IL-6, IL-23, and TNF-α after excluding the infant with congenital illness.

Enhanced inflammation in responses of DENV-infected mothers compared to their children

Analysis of Th17 bias in mothers revealed no suppression in IL-17 from DENV-1 versus OFI ($p = 0.21$; Figure 4A). However, while the inflammatory response in DENV-1 infected infants and young children was unaffected, the Th17 promoting pro-inflammatory cytokine IL-6 was enhanced in DENV-stimulated PBMC from mothers with confirmed dengue infection compared to those incubated with placebo ($p = 0.028$; Figure 4C).

Discussion

The city of Oran is located in the province of Salta, Argentina, 32 km south from the Bolivian border. With a population of 67 000 inhabitants, Oran is the second urban center in the province, located near large rivers in a tropical region. During 2009, 26 923 cases of acute dengue illness due to DENV-1 were detected in Argentina.[7] The dengue outbreak started in Oran, Salta, on epidemiological week 53 in 2008 and finished on epidemiological week 21 in 2009. The dengue outbreak was widespread in the province of Salta with an attack rate of 22/10 000 inhabitants and a mortality rate of 7.46/10 000 cases. Two deaths were reported in adults in Salta: a man of 65 years in Tartagal and a woman of 48 years in Oran. This

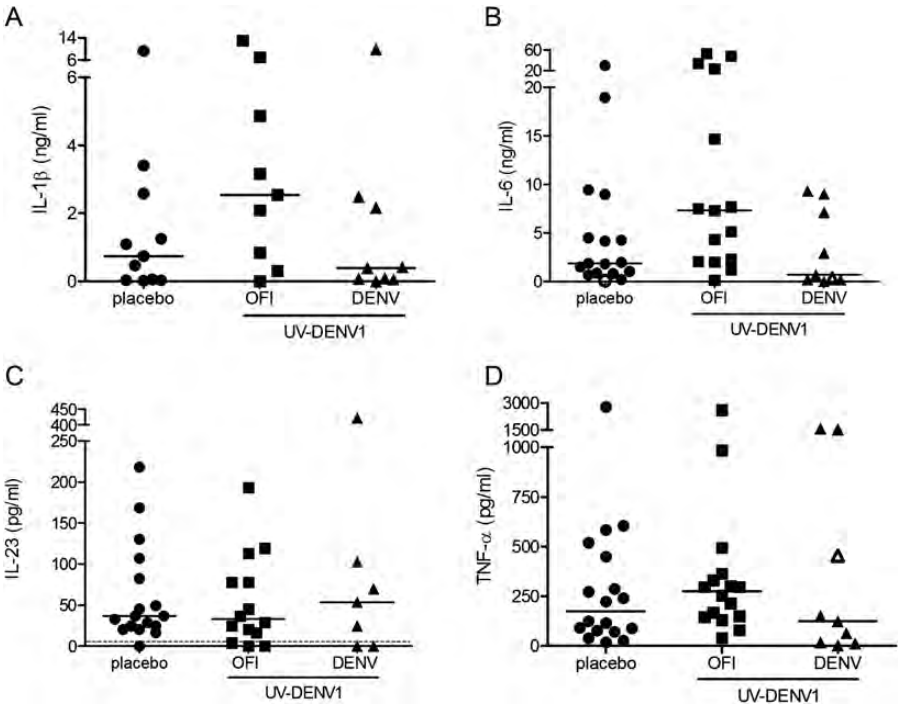

Figure 3. Inflammatory production in dengue infected infants. (A–D) Cytokine levels in supernatant fluids of PBMCs from DENV1-infected infants and young children (triangles, circles) or those presenting OFI (squares, circles). PBMCs were incubated with UV-inactivated DENV1 (UV-DENV1) or placebo. (A) IL-1β, (B) IL-6, (C) IL-23 and (D) TNF-α were analyzed in the supernatants. Lines represent median values. Dashed lines indicate minimum detectable levels for each cytokine. For IL-1β, IL-6, IL-23 and TNF- α, the minimum detectable levels were 1 pg/ml, 2 pg/ml, 6.8 pg/ml, and 1.7 pg/ml, respectively. The infant with congenital illness, whose mother had secondary DENV infection, is indicated with open figures.

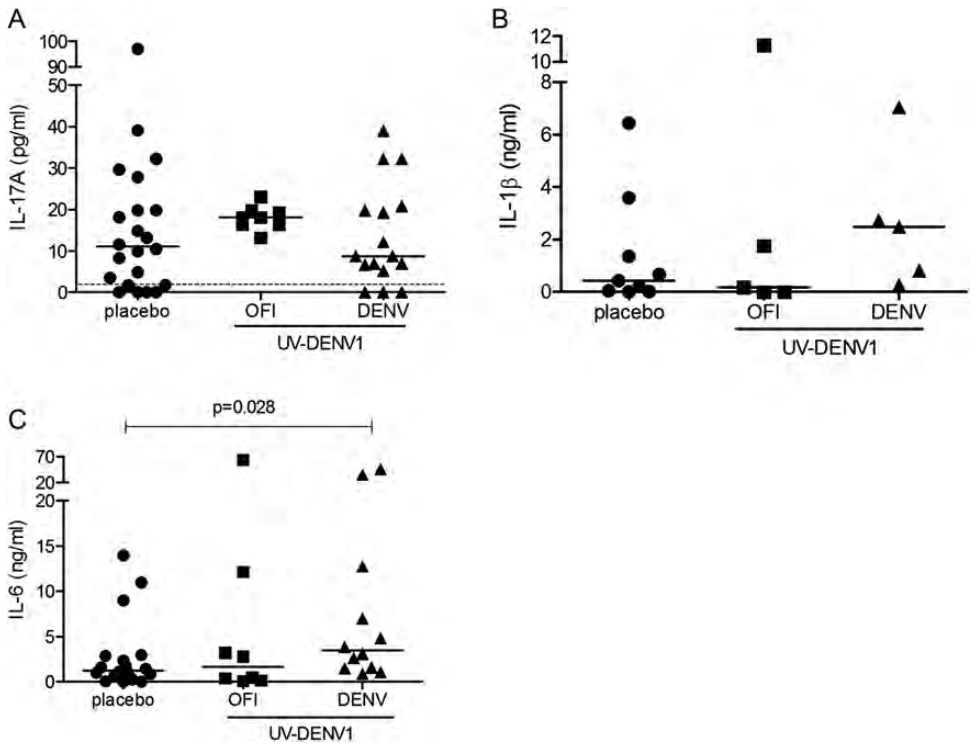

Figure 4. Inflammation in DENV-infected mothers. (A–C) Cytokine levels in supernatant fluids of PBMCs from DENV1- infected (triangles, circles) or OFI (squares, circles) mothers. PBMCs were incubated with UV-inactivated DENV1 (UV-DENV1) or placebo. (A) IL-17A, (B) IL-1β, and (C) IL-6 were measured in the supernatants. Lines represent median values. Dashed lines indicate minimum detectable levels for each cytokine. For IL-17A, IL-1β and IL-6 the minimum detectable levels were 2 pg/ml, 1 pg/ml, and 2 pg/ml, respectively.

outbreak had the highest number of confirmed DENV cases in the region since the re-introduction of DENV in Argentina in 1997.

In our study, conducted in 54 subjects during the 2009 epidemic, we show that adaptive and inflammatory responses to dengue virus infection are different in infants and young children compared to their mothers. This analysis, conducted in a predominantly naïve environment for dengue, evidenced Th2 polarization and milder inflammatory responses in the pediatric immune response to infection compared to adults.

These age-related differences stress the need to better understand immaturity of immune responses during DENV infections in infants. Interestingly, in absence of transplacentally-acquired humoral immunity, most children's disease was mild and only the youngest infants, infected in utero or early in life, required hospitalization. However, these ill children presented a particularly strong Th2 bias (sickest patients had IL-4 levels of 54 pg/ml and 17.5 pg/ml). Previous studies have reported a role for Th2 polarization in the pathogenesis of DHF.[21–25] Altered Th1 responses with low levels of the T-bet transcription factor, suppression of IFN-γ production, lack of IL-12, low IFN-γ/IL-4 ratios, and Fc-γ receptor-mediated potentiation of Th2 responses[21–25] during illness support a role for type 2 bias in disease. However, some of our patients without DHF presented with Th2 polarization. The role of Th2 bias in hemorrhagic presentations requires further study. After adjusting using Bonferroni, IL-4 production was still significantly higher in DENV infected patients vs those with OFI. While it is difficult in the context of a study designed to characterize immune profiles to speculate about the clinical implications of such differences, results support larger studies to address this issue. To our knowledge, this is the first description of Th17 suppression in infants with dengue.

Our findings suggest new roads for the study of severe dengue pathogenesis. First, we show that, contrary to current hypotheses,[21–25] Th2 polarization can be observed in infants with mild disease. Yet, while Th2 bias is not exclusive of severe cases, we also show that Th2 polarization is enhanced in extreme cases suggesting that dengue severity may be a continuum (as hypothesized by other authors).[26] But these grades of severity may be independent of ADE, and Th2 responses may[27,28] or may not associate with disease enhancement mediated by antibodies. The immune system is suspected to enhance or decrease severity of illness during many infectious diseases depending on the age of infection: respiratory syncytial virus bronchiolitis, measles, and other respiratory viruses are most severe in young infants (interestingly, severity is frequently observed in association with Th2 polarization).[8,29,30] Conversely, the severity of varicella and severe acute respiratory syndrome (SARS) increases with age.[31,32] The role for immune immaturity, senescence or previous exposures to related agents in some of these illnesses requires further investigation.

Our manuscript has limitations, one of which is the small study size. In addition, studying children recovering from a debilitating illness limited our sampling to one time point per patient and to small blood volumes, requiring us to privilege certain cytokines over others in our population and precluding testing of every sample for every cytokine. Absolute cytokine differences appear small but the magnitude of meaningful biological differences in this context is unknown. Furthermore, difficult access to the

study site in the jungle limited reagent availability and forced us to use two strategies for cell stimulation. In addition, we could not perform paired analyses of cytokine production between mothers and children due to sample size limitations (some infants with dengue had mothers with OFI and vice versa). However, the study also has important strengths: it demonstrates a clear difference in adaptive and inflammatory responses to DENV-1 infection between children and their mothers, its design decreases potential genetic and environmental confounding, and provides information on previously unknown Th17 responses and partially explored Th2 polarization against DENV.

Conclusions

Enhanced Th2 responses and decreased inflammation differentiate DENV-infected infants and young children from their DENV-infected mothers. Studying the role of these newly recognized age-related differences in dengue pathogenesis should expand our understanding of mechanism of illness in the most prevalent arthropod-borne disease in humans.

Authors' contributions: LBT, JB, VWi and FPP conceived the study; LBT, GAM and FPP designed the study protocol; JB, MAE, MOQ, MB, LEC, DM, GF, and RL carried out the clinical assesments; FAR contributed with materials; LBT, JB, VWi, JPB, PLA, NR and GAM carried out the experiments; LBT, JB, VWi, DRH, VWu, RL and FPP carried out the analysis and interpretation of the data; LBT, VWi, and FPP drafted the manuscript; LBT and FPP critically revised the manuscript. All authors approved the final manuscript. The authors wish it to be known that, in their opinion, LBT, JB and VWi should be regarded as joint first authors. LBT is guarantor of the paper.

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Competing interests: None declared.

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References

1 Gubler DJ. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. Trends Microbiol 2002;10:100–3.

2 Kalayanarooj S, Vaughn DW, Nimmannitya S et al. Early clinical and laboratory indicators of acute dengue illness. J Infect Dis 1997;176: 313–21.

3 Kittigul L, Pitakarnjanakul P, Sujirarat D et al. The differences of clinical manifestations and laboratory findings in children and adults with dengue virus infection. J Clin Virol 2007;39:76–81.

4 Chau TN, Quyen NT, Thuy TT et al. Dengue in Vietnamese infants – results of infection-enhancement assays correlate with age-related disease epidemiology, and cellular immune responses correlate with disease severity. J Infect Dis 2008;198:516–24.

5 Halstead SB, Lan NT, Myint TT et al. Dengue hemorrhagic fever in infants: research opportunities ignored. Emerg Infect Dis 2002;8:1474–9.

6 Kliks SC, Nimmanitya S, Nisalak A et al. Evidence that maternal dengue antibodies are important in the development of dengue hemorrhagic fever in infants. Am J Trop Med Hyg 1988;38:411–9.

7 Dirección de Epidemiología, sala de situación de salud (año 2009). Ministerio de Salud de la Nación Argentina.

8 Munoz FM. Influenza virus infection in infancy and early childhood. Paediatr Respir Rev 2003;4:99–104.

9 Tregoning JS, Schwarze J. Respiratory viral infections in infants: causes, clinical symptoms, virology, and immunology. Clin Microbiol Rev 2010;23:74–98.

10 Roux X, Remot A, Petit-Camurdan A et al. Neonatal lung immune responses show a shift of cytokines and transcription factors toward Th2 and a deficit in conventional and plasmacytoid dendritic cells. Eur J Immunol 2011;41:2852–61.

11 Duangchinda T, Dejnirattisai W, Vasanawathana S et al. Immunodominant T–cell responses to dengue virus NS3 are associated with DHF. Proc Natl Acad Sci U S A 2010;107:16922–7.

12 Mongkolsapaya J, Dejnirattisai W, Xu XN et al. Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. Nat Med 2003;9:921–7.

13 Lanciotti RS, Calisher CH, Gubler DJ et al. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J Clin Microbiol 1992;30:545–51.

14 Balmaseda A, Standish K, Mercado JC et al. Trends in patterns of dengue transmission over 4 years in a pediatric cohort study in Nicaragua. J Infect Dis 2010;201:5–14.

15 Cockburn JJ, Navarro Sanchez ME, Fretes N et al. Mechanism of dengue virus broad cross–neutralization by a monoclonal antibody. Structure 2012;20:303–14.

16 Cockburn JJ, Navarro Sanchez ME, Goncalvez AP et al. Structural insights into the neutralization mechanism of a higher primate antibody against dengue virus. EMBO J 2012;31:767–79.

17 Bugna J, Moreno J, Sanchez S et al. Fever and bleeding in a newborn baby. Pediatr Infect Dis J 2010;29:1153–8.

18 van de Veerdonk FL, Gresnigt MS, Kullberg BJ, et al. Th17 responses and host defense against microorganisms: an overview. BMB Rep 2009;42:776–87.

19 Miossec P, Korn T, Kuchroo VK. Interleukin-17 and type 17 helper T cells. N Engl J Med 2009;361:888–98.

20 Sierra B, Perez AB, Vogt K et al. Secondary heterologous dengue infection risk: Disequilibrium between immune regulation and inflammation? Cell Immunol 2010;262:134–40.

21 Chaturvedi UC. Shift to Th2 cytokine response in dengue haemorrhagic fever. Indian J Med Res 2009;129:1–3.

22 Chen RF, Liu JW, Yeh WT et al. Altered T helper 1 reaction but not increase of virus load in patients with dengue hemorrhagic fever. FEMS Immunol Med Microbiol 2005;44:43–50.

23 Halstead SB, Mahalingam S, Marovich MA et al. Intrinsic antibody-dependent enhancement of microbial infection in macrophages: disease regulation by immune complexes. Lancet Infect Dis 2010; 10:712–22.

24 Pacsa AS, Agarwal R, Elbishbishi EA et al. Role of interleukin-12 in patients with dengue hemorrhagic fever. FEMS Immunol Med Microbiol 2000;28:151–5.

25 Yang KD, Yeh WT, Yang MY et al. Antibody-dependent enhancement of heterotypic dengue infections involved in suppression of IFNgamma production. J Med Virol 2001;63:150–7.

26 Halstead SB. Dengue. Lancet 2007;370:1644–52.

27 Delgado MF, Coviello S, Monsalvo AC et al. Lack of antibody affinity maturation due to poor Toll–like receptor stimulation leads to enhanced respiratory syncytial virus disease. Nat Med 2009;15:34–41.

28 Polack FP, Auwaerter PG, Lee SH et al. Production of atypical measles in rhesus macaques: evidence for disease mediated by immune complex formation and eosinophils in the presence of fusion-inhibiting antibody. Nat Med 1999;5:629–34.

29 Garcia C, Soriano-Fallas A, Lozano J et al. Decreased innate immune cytokine responses correlate with disease severity in children with respiratory syncytial virus and human rhinovirus bronchiolitis. Pediatr Infect Dis J 2011;vol no,pp-pp.

30 Ma J, Dushoff J, Earn DJ. Age-specific mortality risk from pandemic influenza. J Theor Biol 2011;288:29–34.

31 Gilden DH, Kleinschmidt-DeMasters BK, LaGuardia JJ et al. Neurologic complications of the reactivation of varicella-zoster virus. N Engl J Med 2000;342:635–45.

32 Gomersall CD, Joynt GM, Lam P et al. Short-term outcome of critically ill patients with severe acute respiratory syndrome. Intensive Care Med 2004;30:381–7.