Characterization of Type I Interferon Responses in Dengue and Severe Dengue in Children in Paraguay

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Abstract

Background: Infection with dengue virus (DENV) produces a wide spectrum of clinical illness ranging from asymptomatic infection to mild febrile illness, and to severe forms of the disease. Type I interferons (IFNs) represent an initial and essential host defense response against viruses. DENV has been reported to trigger a robust type I IFN response; however, IFN-α/β profile in the progression of disease is not well characterized.

Objectives and study design: In this context, we conducted a retrospective study assessing the circulating serum levels of type I IFNs and related cytokines at different phases of illness in children during the 2011 outbreak of DENV in Paraguay. Demographic, clinical, laboratory and virological data were analyzed.

Results: During defervescence, significantly higher levels of IFN-β, IL-6 and MIP-1β, were detected in severe vs. non-severe dengue patients. Additionally, a significant positive correlation between INF-α and viremia was detected in children with severe dengue. Significant positive correlations were also observed between IFN-β serum levels and hematocrit/hemoglobin during the febrile phase, whereas IFN-α levels negatively correlated with white blood cells during defervescence in severe dengue patients. Furthermore, previous serologic status of patients to DENV did not influence type I IFN production.

Conclusions: The distinct type I IFN profile in children with dengue and severe dengue, as well as its association with viral load, cytokine production and laboratory manifestations indicate differences in innate and adaptive immune responses that should be investigated further in order to unveil the association of immunological and physiological pathways that underlie in DENV infection.

Keywords

Dengue virus; severe dengue; immune response; type I interferon; interferon alpha; interferon beta

1. Background

Dengue is the most rapidly spreading mosquito-borne viral disease worldwide. It is caused by any of the four dengue virus (DENV) serotypes which are transmitted to humans by the bite of *Aedes spp.* mosquitoes [1, 2]. Infection with DENV can remain asymptomatic or cause a spectrum of clinical manifestations of varying severity, ranging from mild fever, formerly known as dengue fever (DF), to a potentially life-threatening disease, characterized by plasma leakage and hemorrhage (dengue hemorrhagic fever [DHF]) leading to hypovolemic shock [3]. In 2009 the World Health Organization proposed a new classification of the disease, which includes dengue with and without warning signs and severe dengue, in order to improve triage and clinical management [4].

Patients with severe dengue manifestations have been reported to present a "cytokine storm" with high levels of circulating cytokines and chemokines [5]. Elevated levels of various cytokines, including interleukins (IL) associated with inflammation and Th2 bias, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, macrophage migration inhibitory factor (MIF) and several chemokines, such as monocyte chemoattractant protein (MCP-1), macrophage inflammatory protein (MIP-1β), RANTES and IP-10 have been reported in patients with severe dengue disease when compared to non-severe cases [6-11]. The imbalance between pro-inflammatory and anti-inflammatory cytokines is thought to induce the malfunction of endothelial cells which consequently leads to plasma leakage in patients with severe dengue disease [12]. The proposed secreted markers of dengue disease progression or severity and their association with viremia are unclear and reports describe contradictory results [8, 13-17].

Type I interferons (IFN-α/β) are often secreted very early after viral infection and provide the first-line of defense against viruses by the induction of an antiviral state in uninfected cells [18, 19]. DENV has been reported to trigger a robust IFN-α/β response, and DENV nonstructural proteins have been found to down-regulate the IFN pathway in humans, inhibiting IFN-regulated gene expression [20-23].

2. Objectives

In the current study, we aimed to evaluate the association of type I IFNs and a selective number of related cytokines with dengue disease severity, viremia and clinical /laboratory parameters in children in Asunción, Paraguay, to understand the interplay of immune mediators involved in severe dengue pathogenesis.

3. Study design

3.1. Study population

Seventy-seven children under 15 years of age were admitted to the Institute of Tropical Medicine (ITM) in Asuncion, Paraguay, with laboratory-confirmed dengue during a 2011 DENV-1 and DENV-2 outbreak and were included in this study.

Demographic and clinical data were recorded on standardized case report forms during hospitalization. Blood samples were collected during the febrile phase (2-7 days) or defervescence (8-11 days) for hematological and biochemical laboratory tests, serological and/or virological DENV infection, and cytokine determinations.

3.2. Ethics statement

The protocol for this study was approved by the Institutional Review Board of the ITM. Parents or legal guardians of all subjects provided written informed consent, and subjects 8 years of age and older provided assent. The confidentiality of patients was conserved for data analysis. This study is consistent with the principles outlined in the Declaration of Helsinki.

3.3. Sera isolation and identification of DENV positive patients

Sera were collected by centrifugation of blood-containing tubes at 1500 rpm for 10 minutes and the serum samples were stored at -80°C until further use. DENV infection was

confirmed by one or more of the following methods: (i) reverse transcription polymerase chain reaction (RT-PCR) amplification of viral RNA [24] (ii) DENV NS1 antigen determination by immunocromatography (Bioeasy™, Standard Diagnostics INC, Korea), (iii) the presence of IgM and/or IgG by ELISA (SD Dengue IgM and IgG Capture ELISA, Standard Diagnostics INC, Korea) in paired acute and convalescent-phase serum samples, as previously described [25]. The PanBio Dengue IgG Capture ELISA was used for the determination of pre-existing immunity to DENV, according to the manufacturer´s instructions.

3.4. Viremia quantification

RNA was extracted from serum samples during the febrile phase using QIAamp Viral RNA Minikit (QIAGEN). The amount of viral RNA in serum was quantified by using StepOnePlus Real-Time PCR System (Applied Biosystems) employing TaqMan technology [26]. Standard curves were generated using 10-fold serial dilutions of viral RNA obtained from purified DENV-1 or DENV-2 suspensions.

3.5. Cytokine quantification

Type I IFNs were determined in serum samples from the febrile phase and defervescence using Verikine™ Human IFN Alpha and Beta ELISA Kits (Pestka Biomedical Laboratories Inc., Piscataway, NJ, USA) following the manufacturer´s instructions. Additional cytokines were assayed in serum samples using Bio-Plex Pro™ Human Cytokine 17-plex Assay (Bio-Rad Laboratories Inc., Hercules, CA, USA). Measurements were performed using the Bio-Plex MAGPIX Multiplex Reader (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer´s instructions.

3.6. Statistical analysis

Data were analyzed using the chi square test for categorical variables and the non-parametric Mann-Whitney test for continuous variables. Univariate logistic regression analyses were performed to investigate the association of relevant independent variables

with the outcomes (dengue *vs.* severe dengue) and odds ratios were calculated where appropriate. The correlation between cytokine levels and laboratory parameters was performed calculating the Spearman correlation coefficient, r_s (ranging from -1 to 1). A p value <0.05 was considered significant for all the tests performed. Statistical analyses were performed using Stata/SE 13.0 for Windows (StataCorp LP, College Station, TX, USA).

4. Results

4.1. Characteristics of the study population

Of the 77 children under the age of 15 years who were admitted to the ITM with laboratory-confirmed dengue during a 2011 DENV outbreak in Paraguay, 16 children (20.8%) presented dengue illness (DI) and 61 children (79.2%) presented severe dengue (SD), according to the WHO 2009 guidelines (Table 1). The laboratory diagnostic tests performed are shown in Table S1. Patients with DI included children with (n=14) and without (n=2) warning signs. The demographics and clinical characteristics of children with DI or SD are summarized in Table 1. The mean age of patients was similar between both groups. In addition, no significant differences were identified in sex distribution and weight between groups. Patients were admitted at 3.8±1.7 and 3.5±1.8 days after the onset of symptoms for DI and SD patients, respectively. The duration of hospitalization was significantly higher in SD compared to DI children and eight patients were admitted to the intensive care unit.

Twenty-five patients presented clinical complications, including hepatitis, polyserositis and miocarditis (Table 1). Comorbidities were detected in both DI and SD groups, and included being overweight or having choledochian syndrome, recurrent wheezing or convulsions (Table 1). One death was reported in the SD group.

The most frequent clinical manifestations among DI and SD patients were fever, myalgia and arthralgia, headache, vomiting and abdominal pain (Table 2). In addition, SD children developed a variety of other symptoms, including hypotension, encephalitis, ascites, pleural effusion, gallbladder wall thickening and pericardial effusion (Table 2). The duration of

hospitalization was significantly associated with SD (OR 1.446, 95% CI 1.047-1.999; p = 0.025).

SD patients exhibited significantly lower albumin in blood and prolonged APTT compared to DI patients (Table 3). All SD and DI patients with warning signs required intravenous fluid therapy while hospitalized, while a small proportion of patients received platelet transfusions, mechanical ventilation, inotropic therapy and antibiotics.

4.2. Analysis of pre-existing immunity to DENV in the study population

When previous exposure to DENV was analyzed in the study population, we found that 25 and 48 patients had primary and secondary DENV infections, respectively. Interestingly, no significant differences in dengue severity were observed between these two groups of patients (Table 4). Demographic and clinical characteristic comparisons as well as the laboratory profile of both groups of patients are shown in Tables S2-S4. The analysis of 19 different types of cytokines, including inflammatory-, Th- and macrophage related-cytokines, chemokines and interferons in serum samples from patients did not show significant differences between patients with primary and secondary infections, with the exception of a significant increase in IL-β levels and a trend towards augmented IL-6 and IL-8 levels in patients with secondary infections, suggesting a moderately enhanced inflammatory response in this group of children (Table S5). Due to the minimal differences in inflammatory profile in patients with primary and secondary infections, we then studied the cytokine profile and a possible association of antiviral mediators –type I IFN– and related cytokines in terms of dengue disease severity.

4.3. Type I IFN levels in DI vs. SD patients at different phases of illness

DENV infection has been reported to induce a type I IFN response in patients. However, the regulation of this response during dengue illness and the association with disease severity are not well characterized [15, 16, 27, 28]. To investigate the progression of type I IFNs in DI and SD patients, we determined IFN-α and IFN-β levels in serum samples

of patients during the febrile phase and defervescence. Significantly higher levels of IFN-β were detected in SD patients compared to DI patients during defervescence (Fig. 1b), while IFN- β levels did not exhibit statistically significant differences between both groups of patients during febrile phase. Despite displaying a similar pattern of responses to that of IFN-β, no significant differences were observed in IFN-α levels during both phases of illness when comparing both groups of children (Fig. 1a).

Several reports have described conflicting results with respect to the role of serum viral load in the severity of dengue disease [29-32]. Therefore, we further investigated a possible association of viremia and plasma levels of type I IFNs in SD patients. The serum DENV viremia levels were measured in 42 total patients during the febrile phase, where available (n = 11 and 31 for DI and SD patients, respectively; n = 18 and 24 for primary and secondary DENV infected patients, respectively). We found a positive correlation between IFN-α and viremia in SD patients (Fig. 1c) suggesting that, as expected, DENV replication induces production of IFN-α. No significant correlation was found between IFN-β and viremia (Fig. 1d). Interestingly, in our study viremia levels were not significantly different in DI and SD children as well as in primary and secondary DENV infected patients (Fig. 2a and 2b).

4.4. Type I IFN-related cytokine levels in DI vs. SD patients at different phases of illness

Type I IFNs can signal to almost every cell type in the body and closely interact with the immune system affecting cytokine and chemokine production [33-35]. Therefore, we then explored the serum levels of type I IFN-related cytokines and chemokines of patients during the febrile phase or defervescence. The significant increase in serum IFN-β levels in SD compared to DI patients during defervescence (Fig. 1b) was accompanied by significantly higher levels of type I IFN-promoted and pro-inflammatory mediators, IL-6 (Fig. 3a) and MIP-1β (Fig. 3b). No statistically significant differences were detected in serum levels of other type I IFN-related mediators, IL-10, MCP-1 and IFN-γ between DI and SD children (Fig. 3c-e).

193 *4.5. Association of type I IFN levels and laboratory parameters in DI and SD patients*

194 We next analyzed a possible association of type I IFN levels and laboratory
195 parameters in DI and SD patients (Fig. 4). During the febrile phase, increased hematocrit
196 levels were associated with augmented levels of IFN-β in SD patients (Fig. 4a). Conversely,
197 no significant correlations were observed between type I IFNs and laboratory parameters
198 during febrile phase in DI patients. Furthermore, during defervescence significant
199 associations between increased levels of WBC and decreased levels of IFN-α were observed
200 in SD patients (Fig. 4b).

201

202 ## 5. Discussion

203 Type I IFN is an important innate immune system regulator of viral infections [18, 33,
204 36]. The role of type I IFN in DENV infection control and disease severity has been studied in
205 vitro and in vivo [37-41]. While DENV infection has been shown to induce a strong type I IFN
206 response in patients, the association with disease severity in clinical studies has yielded
207 mixed results [15, 16, 28, 40, 41]. In our study, we found that SD patients exhibited
208 significantly higher levels of IFN-β during defervescence compared to DI patients, whereas
209 IFN-α levels did not significantly differ between both groups of patients during disease
210 progression.

211 The early studies that explored the role of type I IFN in dengue severity in human
212 subjects reported that similarly high levels of IFN-α were present in serum of Thai children
213 with DHF and DF [40]. However, several subsequent reports from Mexico, Taiwan, Brazil,
214 India and Thailand described higher levels of IFN-α in patients with milder clinical forms of
215 dengue [14, 15, 27, 32, 42]. Although the production of IFN-α in DENV-infected patients and
216 its association with disease severity is well documented, less is known about the role of IFN-
217 β in DENV-infected patients. A study by Pech Torres et al. found that serum IFN-β levels
218 were not associated with severity of dengue disease, reporting that DF and DHF patients as
219 well as dengue patients with and without warning signs expressed similar serum levels of

IFN-β [16]. By contrast and in agreement with our results, another recent study revealed that high levels of IFN-β were associated with primary DHF in patients from Northeast Brazil, whereas IFN-α levels were similarly expressed in DF and DHF patients [43].

The relationship between type I IFN levels and the previous serologic status of patients to DENV is controversial [15, 27, 28, 32, 43]. In our study, we did not observe significant differences in serum levels of IFN-α and IFN-β in patients with primary and secondary infections, suggesting that the pre-existing immunity to DENV in our study population did not influence type I IFN production.

The kinetics of type I IFN induction by DENV infection as well as the main IFN producer-cell type has been reported to be different between IFN-α and-β and may account for the different expression levels observed in serum of DENV-infected patients [28, 44, 45]. Tang et al. reported that in hospitalized DENV-1 infected adult patients from China serum IFN-α levels were increased compared to serum IFN-β levels, which were similar to the ones in healthy controls [28]. Furthermore, while some reports describe that levels of IFN-α decline rapidly after fever onset in DF and DHF patients [15], other studies found that increased levels of IFN-α remain after defervescence in some patients with DHF, but not in patients with DF [40]. In our study, we observed that IFN-α and IFN-β levels remained low and close to baseline in DI patients during the febrile phase and defervescence. By contrast, both type I IFN levels were more dispersed in SD than in DI patients but did not exhibit significant differences during disease progression.

The relationship between viral load and the clinical severity of DENV infection has been described in several reports, which yielded again conflicting results [14, 29, 31, 32, 46-49]. Some reports informed higher viremia levels in DHF than in DF patients [14, 29, 31, 47]. Conversely and in agreement with our study, other studies found similar viral RNA levels in serum samples of patients undergoing mild and severe dengue infections [32, 46, 48, 49]. Furthermore, coinciding with observations from other authors, we observed a positive correlation between dengue viremia and IFN-α in SD patients [28, 32], which was absent with IFN-β. These results and the lack of association of IFN-α levels with disease severity suggest

248 that IFN-α induction by DENV replication is not related with the development of severe
249 manifestations of dengue disease in our study population.
250 Several studies associate SD disease with excessive immune activation that creates
251 a cytokine cascade resulting in increased vascular permeability [50, 51]. In our study, we
252 observed that SD patients exhibited significantly increased serum levels of IL-6 and MIP-1β
253 compared to DI children. IL-6 is secreted primarily by macrophages and lymphocytes in
254 response to injury or infection [52] and, in concordance with our study, has been reported to
255 be associated with the progression from mild dengue to severe forms of the disease [6-8,
256 53]. MIP-1β has been proposed to play a role in immunopathogenesis, by contributing to
257 fever and bone marrow suppression in DENV infections [54]. In a recent study that
258 investigated prognostic biomarkers of DHF, MIP-1β was reported to be significantly
259 increased in DHF compared to DF patients [54]. Conversely, other studies have described
260 MIP-1β as a good prognostic marker of dengue disease [8, 55].
261 Blood profile and laboratory parameters were similar in DI and SD patients in our
262 study population, except for significantly decreased albumin in blood, probably due to liver
263 dysfunction, and increased APTT, related to the risk or the presence of hemorrhage in SD
264 compared to DI children. The association of type I IFN levels and laboratory parameters has
265 not been extensively studied in dengue patients. Serum IFN-α levels have been reported to
266 be directly proportional to the degree of thrombocytopenia, suggesting that IFN-α may inhibit
267 platelet production from human megakaryocytes [28, 40]. Furthermore, IFN-α levels have
268 been described to be negatively correlated with serum alanine aminotransferase levels in DF
269 patients [28]. In our study we found that augmented IFN-β levels were associated with
270 increased hematocrit in SD patients, suggesting that IFN-β may exert an undesirable
271 immune-regulatory effect in DENV-infected patients associated with hemoconcentration. In
272 addition, we found that IFN-α levels negatively correlated with white blood cell count, also
273 suggesting that type I IFN may negatively impact the bone marrow during DENV infections.
274 Our study had several limitations. First, the number of patients with DI was small,
275 potentially hampering detection of weaker associations. Furthermore, due to the availability

of small blood volumes, the association between viremia and type I IFNs could be performed with a limited number of samples. This said, the observations on type I IFNs and related cytokines, such as IL-6 and MIP-1β, describe an important role of IFN-β in severe disease manifestations in children from a Latin American country and provide an interesting perspective of two very different phases of illness in both moderate and severe presentations. Further studies with a larger population of patients would provide a more comprehensive view of type I IFNs associations with dengue disease manifestations, in terms of prognosis or pathophysiology.

In summary, the results of our study highlight the intrincate interaction of type I IFNs in the immune response to DENV infection in humans, and stress the need for more investigation on their effects in dengue disease.

Competing interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Authors´ contributions

Conceived and designed experiments: LBT SA DL CV GC AA FPP. Performed the experiments: LBT ABB SA CV PLA AF MTC. Analyzed the data: LBT ABB SA CV. Wrote the paper: LBT FPP.

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Figure legends

Fig. 1. Type I IFN production and correlation with viremia levels in DENV-infected children. (a) IFN-α and (b) IFN-β levels in serum samples from patients with DI compared to those with SD at different phases of illness (febrile phase or defervescence). Lines represent median values. Correlations between (c) IFN-α and (d) IFN-β levels with viremia during the febrile phase in SD patients. r_s, Spearman correlation coefficient. * $p<0.05$.

Fig. 2. Viremia levels do not significantly differ in DI and SD children and in primary and secondary DENV infected patients. Box-and-whisker plots show median values (horizontal line in the box), 25-75% interquartile range (upper and lower limits of the box), and maximum and minimum values (additional bars).

Fig. 3. Type I IFN-related cytokine production in DENV-infected children over two phases of illness. Type I IFN-related cytokine levels in serum samples from patients with DI compared to those with SD at the febrile phase or defervescence. Lines represent median values. * $p<0.05$.

Fig. 4. Correlations of IFN-α and IFN-β levels with laboratory parameters in SD patients. IFN-β serum levels positively correlated with (a) hematocrit during the febrile phase and IFN-α serum levels negatively correlated with (b) WBC during defervescence in SD patients. r_s, Spearman correlation coefficient. * $p<0.05$.

Table 1. Demographic and clinical characteristics of the study population.

Demographic and clinical characteristics	DI (n=16)	SD (n=61)	p-value[a]	OR (95% CI)	p-value[b]
Age in years (mean, StD)	10.3 (4.6)	10.8 (3.9)	0.757	1.035 (0.906-1.182)	0.615
Age in years (range)	1−15	0−15	−−−	−−−	
Sex			0.935	1.047 (0.345-3.178)	0.935
Female (n, %)	7 (43.7)	26 (42.6)			
Male (n, %)	9 (56.3)	35 (57.4)			
Weight (mean, StD)	39.8 (19.3)	44.0 (16.8)	0.235	1.014 (0.982-1.048)	0.387
Day of disease at hospitalization (mean, StD)	3.8 (1.7)	3.5 (1.8)	0.570	0.921 (0.675-1.256)	0.603
Days of hospitalization (mean, StD)	3.9 (1.8)	5.5 (2.7)	0.019	1.446 (1.047-1.999)	0.025
Day of hospitalization (range)	1−7	1−18	−−−	−−−	
ICU Entry (n, %)	0 (0)	8 (13.1)	0.126	−−−	
Clinical complications[c] (n, %)	2 (12.5)	23 (37.7)	0.055	4.237 (0.882-20.354)	0.071
Comorbidities[d] (n, %)	2 (12.5)	11 (18.0)	0.599	1.54 (0.305-7.774)	0.601
Deaths (n, %)	0 (0)	1 (1.6)	0.606	−−−	

DI, dengue illness; SD, severe dengue; OR, odds ratio; CI, confidence interval; StD, standard deviation; ICU, intensive care unit.

[a] p-values were calculated using Chi-square tests except for age, weight, day of disease at hospitalization and days of hospitalization, for which Mann-Whitney tests were applied.

[b] p-values were calculated during univariate logistic regression analyses using 2-tailed test.

[c] Clinical complications included hepatitis, polyserositis, and miocarditis.

[d] Comorbidities included overweight, choledochian syndrome, recurrent wheezing, and convulsions.

Table 2. Clinical symptoms experienced by the study population.

Clinical symptoms	DI (n=16) n (%)	SD (n=61) n (%)	p-value[a]	OR (95% CI)	p-value[b]
Fever	16 (100)	61 (100)	---	---	---
Rash	6 (37.5)	17 (27.9)	0.454	0.644 (0.203-2.047)	0.456
Myalgia and arthralgia	9 (56.3)	39 (63.9)	0.572	1.377 (0.451-4.215)	0.573
Headache	14 (87.5)	39 (63.9)	0.070	0.253 (0.053-1.219)	0.087
Epistaxis	3 (18.8)	8 (13.1)	0.566	0.654 (0.152-2.814)	0.568
Bleeding gums	2 (12.5)	4 (6.6)	0.430	0.491 (0.082-2.958)	0.438
Metrorrhagia	1 (6.3)	2 (3.3)	0.585	0.508 (0.043-5.990)	0.591
Hematemesis	0 (0)	3 (4.9)	0.366	---	---
Vomiting	10 (62.5)	31 (50.8)	0.405	0.620 (0.200-1.919)	0.407
Abdominal pain	11 (68.8)	38 (62.3)	0.633	0.751 (0.231-2.437)	0.633
Hypotension	0 (0)	3 (4.9)	0.366	---	---
Encephalitis	0 (0)	1 (1.6)	0.606	---	---
Ascites	0 (0)	23 (37.7)	0.003	---	---
Pleural effusion	0 (0)	20 (32.8)	0.008	---	---
Gallbladder wall thickening	1 (6.3)	17 (27.9)	0.069	5.795 (0.709-47.336)	0.101
Pericardial effusion	0 (0)	6 (9.8)	0.191	---	---

DI, dengue illness; SD, severe dengue; OR, odds ratio; CI, confidence interval.

[a] p-values were calculated using Chi-square tests.

[b] p-values were calculated during univariate logistic regression analyses using 2-tailed test.

Table 3. Blood profile and laboratory determinations of the study population.

Laboratory determinations	Normal level	DI	SD	p-value[a]
	(Range)	(n=16)	(n=61)	
		Mean (StD)		
White Blood Cells (x10^6/L)	4000−11000	4618.8 (2643.3)	5096.7 (2925.5)	0.585
Hematocrit (%)	36−50	39.9 (6.5)	40.0 (6.2)	0.422
Hemoglobin (g/dL)	12.1−17.2	12.8 (1.3)	13.5 (1.6)	0.205
Platelets (x10^6/mL)	150−400	202.0 (137.5)	161.4 (88.5)	0.451
Aspartate aminotransferase, AST (IU/L)	10−34	114.8 (87.1)	185.1 (349.8)	0.797
Alanine aminotransferase, ALT (IU/L)	7−40	82.7 (69.1)	109.9 (204.5)	0.758
Total Albumin (g/dL)	3.4−5.4	4.3 (0.6)	3.4 (0.4)	0.034
Plasma Urea (mg/dL)	7−20	23.5 (7.5)	21.3 (8.4)	0.427
Creatinine (mg/dL)	0.5−1.2	0.55 (0.17)	0.57 (0.17)	0.779
Sodium (mEq/L)	135−145	139.0	136.7 (5.6)	0.560
Potassium (mEq/L)	3.7−5.2	4.0	4.03 (0.45)	0.678
Prothrombin Time, PT (%)	70−130	99.5 (0.7)	83.4 (12.8)	0.117
Activated Partial Thromboplastin Time, APTT (seg)	25−35	26.5 (3.5)	36.7 (7.9)	0.048
Fibrinogen (mg/dL)	200−400	350.0 (45.3)	307.6 (94.3)	0.472

DI, dengue illness; SD, severe dengue; StD, standard deviation.

[a] p-values were calculated using Mann-Whitney tests.

Table 4. Pre-existing immunity to DENV infection in the study population.

Type of infection	DI	SD	p-value[a]	OR (95% CI)	p-value[b]
	(n=16)	(n=61)			
Primary infection[c] (n, %)	6 (37.5)	19 (31.2)	0.629	0.754 (0.239-2.377)	0.630
Secondary infection[d] (n, %)	9 (56.3)	39 (63.9)	0.572	1.379 (0.451-4.215)	0.573
Not classified[e] (n, %)	1 (6.3)	3 (4.9)	---	---	---

DI, dengue illness; SD, severe dengue; OR, odds ratio; CI, confidence interval.

[a] p-values were calculated using Chi-square tests.

[b] p-values were calculated during univariate logistic regression analyses using 2-tailed test.

[c] Samples with (OD/CV) x 10 < 18, where OD is the absorbance of the test sample and CV is the Cutt-off Value in IgG Capture ELISA.

[d] Samples with (OD/CV) x10 > 22 in IgG Capture ELISA.

[e] Samples with (OD/CV) x 10 in the range 18-22 in IgG Capture ELISA.

Supplementary tables

Table S1. Laboratory test results in the study population.

Laboratory diagnostic tests	DI (n=16)	SD (n=61)
	n	
DENV RNA detection	11	31
DENV NS1 Antigen detection	1	3
DENV IgM detection	15	56
Multiple DENV NS1 Antigen/IgM detection	1	3
Multiple DENV RNA /IgM detection	10	30
DENV IgG detection	16	61
Primary infections[a]	6	19
Secondary infections[b]	9	39
Not classified[c]	1	3

DI, dengue illness; SD, severe dengue.

[a] Samples with (OD/CV) x 10 < 18, where OD is the absorbance of the test sample and CV is the Cutt-off Value in IgG Capture ELISA.

[b] Samples with (OD/CV) x10 > 22 in IgG Capture ELISA.

[c] Samples with (OD/CV) x 10 in the range 18-22 in IgG Capture ELISA.

Table S2. Demographic and clinical characteristics of primary and secondary DENV infected patients.

Demographic and clinical characteristics	Primary infection	Secondary infection	p-value[a]
	(n=25)	(n=48)	
Age in years (mean, StD)	9.2 (4.5)	11.3 (3.7)	0.047
Age in years (range)	0 - 15	1 - 15	---
Sex			0.234
Female (n, %)	13 (52.0)	18 (37.5)	
Male (n, %)	12 (48.0)	30 (62.5)	
Weight (mean, StD)	37.0 (18.4)	45.7 (16.5)	0.080
Day of disease at hospitalization (mean, StD)	3.2 (1.7)	3.6 (1.7)	0.264
Days of hospitalization (mean, StD)	5.4 (3.3)	5.1 (2.3)	0.628
Day of hospitalization (range)	1 - 18	1 - 15	---
ICU Entry (n, %)	2 (8.0)	5 (10.4)	0.739
Clinical complications (n, %)	4 (16.0)	20 (41.7)	0.027
Comorbidities (n, %)	3 (12.0)	10 (20.8)	0.349
Deaths (n, %)	0 (0)	1 (2.1)	0.467

StD, standard deviation; ICU, intensive care unit.

[a] p-values were calculated using Chi-square tests except for age, weight, day of disease at hospitalization and days of hospitalization, for which Mann-Whitney tests were applied.

Table S3. Clinical symptoms experienced by primary and secondary DENV infected patients.

Clinical symptoms	Primary infection	Secondary infection	p-value[a]
	(n=25)	(n=48)	
	n (%)	n (%)	
Fever	25 (100)	48 (100)	---
Rash	9 (36.0)	14 (29.2)	0.551
Myalgia and arthralgia	10 (40.0)	35 (72.9)	0.006
Headache	15 (60.0)	34 (70.8)	0.350
Epistaxis	2 (8.0)	9 (18.8)	0.223
Bleeding gums	0 (0.0)	5 (10.4)	0.095
Metrorrhagia	2 (8.0)	0 (0.0)	0.047
Hematemesis	0 (0.0)	3 (6.3)	0.202
Vomiting	13 (52.0)	25 (52.1)	0.995
Abdominal pain	13 (52.0)	34 (70.8)	0.111
Hypotension	0 (0.0)	2 (4.2)	0.301
Encephalitis	0 (0.0)	1 (2.1)	0.467
Ascites	4 (16.0)	18 (37.5)	0.057
Pleural effusion	2 (8.0)	17 (35.4)	0.011
Gallbladder wall thickening	2 (8.0)	15 (31.3)	0.026
Pericardial effusion	1 (4.0)	5 (10.4)	0.344

[a] p-values were calculated using Chi-square tests.

Table S4. Blood profile and laboratory determinations in primary and secondary DENV infected patients.

Laboratory determinations	Normal level (Range)	Primary infection (n=25)	Secondary infection (n=48)	p-value[a]
		Mean (StD)		
White Blood Cells (x10^6/L)	4000-11000	5448.0 (3314.8)	4893.8 (2675.2)	0.839
Hematocrit (%)	36-50	38.0 (5.0)	40.8 (6.7)	0.041
Hemoglobin (g/dL)	12.1-17.2	12.7 (1.6)	13.6 (1.5)	0.043
Platelets (x10^6/mL)	150-400	211.1 (121.8)	145.6 (77.7)	0.011
Aspartate aminotransferase, AST (IU/L)	10-34	125.0 (105.0)	204.9 (395.7)	0.537
Alanine aminotransferase, ALT (IU/L)	7-40	86.3 (78.9)	119.0 (229.9)	0.853
Total Albumin (g/dL)	3.4-5.4	3.6 (0.3)	3.5 (0.5)	0.435
Plasma Urea (mg/dL)	7-20	23.0 (9.9)	21.1 (7.5)	0.867
Creatinine (mg/dL)	0.5-1.2	0.6 (0.2)	0.6 (0.1)	1.000
Sodium (mEq/L)	135-145	134.4 (8.7)	138.2 (2.6)	0.689
Potassium (mEq/L)	3.7-5.2	3.8 (0.5)	4.1 (0.4)	0.233
Prothrombin Time, PT (%)	70-130	91.4 (12.8)	81.7 (12.3)	0.087
Activated Partial Thromboplastin Time, APTT (seg)	25-35	30.5 (5.8)	38.8 (8.1)	0.037
Fibrinogen (mg/dL)	200-400	335.8 (93.3)	290.8 (91.0)	0.440

StD, standard deviation.

[a] p-values were calculated using Mann-Whitney tests.

Table S5. Cytokine profile at different phases of illness in primary and secondary DENV infected patients.

Inflammatory cytokines and interferons	Febrile phase[a]			Defervescence[b]		
	Primary infection (n=19)	Secondary infection (n=28)	p-value[c]	Primary infection (n=6)	Secondary infection (n=20)	p-value[c]
	(pg/ml) Median (IQR)			(pg/ml) Median (IQR)		
IL-1β	0.0 (0.0-0.6)	1.3 (0.0-7.4)	0.0210	0.3 (0.0-0.6)	0.3 (0.3-0.5)	0.9421
IL-2	11.5 (0.0-18.9)	16.5 (5.5-27.9)	0.1453	12.1 (12.1-13.1)	13.6 (7.2-23.0)	0.7074
IL-6	9.4 (5.6-14.0)	14.5 (7.5-39.4)	0.0560	23.2 (11.3-25.9)	13.6 (7.1-18.4)	0.3582
IL-7	6.0 (1.3-13.7)	8.0 (1.3-17.2)	0.5936	8.0 (6.0-8.0)	4.3 (3.8-12.7)	0.6795
IL-8	8.9 (2.8-17.8)	21.9 (5.1-55.3)	0.0709	27.5 (5.8-63.2)	13.0 (9.0-25.2)	0.7856
IL-17A	4.9 (3.0-16.1)	9.6 (4.9-21.7)	0.3618	10.5 (4.9-30.9)	12.4 (5.8-18.0)	0.7850
TNF-α	0.0 (0.0-3.3)	0.0 (0.0-21.2)	0.4633	0.0 (0.0-3.3)	0 (0-3.3)	0.8037
IFN-α	0.6 (0.0-19.9)	2.0 (0.0-12.3)	0.8300	0.0 (0.0-0.0)	0.0 (0.0-5.9)	0.3574
IFN-β	3.6 (0.0-18.8)	1.0 (0.0-33.5)	0.9524	0.1 (0.0-18.8)	2.4 (0.0-18.8)	0.9160
G-CSF	11.9 (7.8-19.5)	11.9 (9.9-23.2)	0.4038	11.9 (7.8-19.5)	11.9 (7.8-15.8)	0.7239
Th 1 and 2 cytokines						
IFN-γ	36.7 (11.3-58.8)	47.7 (11.3-171.8)	0.3922	36.7 (11.3-36.7)	42.3 (36.7-79.4)	0.0962
IL-12p70	1.2 (1.2-4.4)	2.8 (1.2-7.7)	0.5039	1.2 (1.2-2.8)	2.8 (1.2-2.8)	0.6968
IL-4	1.1 (0.6-2.3)	1.3 (1.0-1.7)	0.5766	1.0 (0.6-1.3)	1.1 (1.0-1.7)	0.5766
IL-5	5.3 (0.1-8.4)	5.3 (0.1-15.8)	0.3846	5.3 (5.3-5-3)	5.3 (0.1-5.3)	0.3402
IL-13	2.9 (0.4-7.8)	4.7 (0.4-6.3)	0.3530	2.9 (2.9-4.7)	2.9 (0.4-3.8)	0.4787
Macrophage related cytokines and chemokines						
GM-CSF	131.1 (69.5-179.8)	131.6 (76.0-201.5)	0.7650	119.7 (51.9-130.4)	117.5 (0-188.3)	0.7324
MIP-1β	58.1 (42.6-106.6)	91.8 (31.0-161.5)	0.5502	165.8 (62.3-193.2)	96.3 (40.0-133.8)	0.1741
MCP-1	71.2 (47.2-197.2)	139.2 (76.6-252.9)	0.0911	101.1 (65.6-150.9)	96.-5 (65.6-125.1)	0.9457
IL-10	8.3 (3.7-16.7)	6.0 (3.7-19.3)	0.9358	4.6 (4.1-6.4)	4.6 (3.0-5.3)	0.4952

IQR, interquartile range. [a] Febrile phase is defined within 2 to 7 days from the onset of symptoms. [b] Defervescence, is defined within 8 to 11 days from the onset of symptoms.[c] p-values were calculated using Mann-Whitney tests.