

RESEARCH ARTICLE

# Quantification of dengue virus specific T cell responses and correlation with viral load and clinical disease severity in acute dengue infection

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**Citation:** Wijeratne DT, Fernando S, Gomes L, Jeewandara C, Ginneliya A, Samarasekara S, et al. (2018) Quantification of dengue virus specific T cell responses and correlation with viral load and clinical disease severity in acute dengue infection. *PLoS Negl Trop Dis* 12(10): e0006540. <https://doi.org/10.1371/journal.pntd.0006540>

**Editor:** Sujan Shresta, La Jolla Institute for Allergy and Immunology, UNITED STATES

**Received:** May 15, 2018

**Accepted:** August 20, 2018

**Published:** October 1, 2018

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**Data Availability Statement:** All relevant data are within the paper and its Supporting Information files.

**Funding:** Funding was provided by the Centre for Dengue Research, University of Sri Jayewardenepura, National Science Foundation, Sri Lanka (NSF/RG/2016/HS/05) and by the Medical Research Council (UK). GSO receives support from the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre (BRC). The

## Abstract

### Background

In order to understand the role of dengue virus (DENV) specific T cell responses that associate with protection, we studied their frequency and phenotype in relation to clinical disease severity and resolution of viraemia in a large cohort of patients with varying severity of acute dengue infection.

### Methodology/Principal findings

Using *ex vivo* IFN $\gamma$  ELISpot assays we determined the frequency of dengue viral peptide (DENV)-NS3, NS1 and NS5 responsive T cells in 74 adult patients with acute dengue infection and examined the association of responsive T cell frequency with the extent of viraemia and clinical disease severity. We found that total DENV-specific and DENV-NS3-specific T cell responses, were higher in patients with dengue fever (DF), when compared to those with dengue haemorrhagic fever (DHF). In addition, those with DF had significantly higher ( $p = 0.02$ ) DENV-specific T cell responses on day 4 of infection compared to those who subsequently developed DHF. DENV peptide specific T cell responses inversely correlated with the degree of viraemia, which was most significant for DENV-NS3 specific T cell responses (Spearman's  $r = -0.47$ ,  $p = 0.0003$ ). The frequency of T cell responses to NS1, NS5 and pooled DENV peptides, correlated with the degree of thrombocytopenia but had no association with levels of liver transaminases. In contrast, total DENV-IgG inversely correlated with the degree of thrombocytopenia and levels of liver transaminases.

### Conclusions/Significance

Early appearance of DENV-specific T cell IFN $\gamma$  responses before the onset of plasma leakage, appears to associate with milder clinical disease and resolution of viraemia, suggesting a protective role in acute dengue infection.

funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing interests:** The authors have declared that no competing interests exist.

## Author summary

In order to understand the role of dengue virus (DENV) specific T cell responses in protection against infection, we studied T cell cytokine production in relation to clinical disease severity and resolution of viraemia in a large cohort of patients with varying severity of acute dengue infection. We found that DENV-specific T cell responses were higher in patients with dengue fever, when compared to those with dengue haemorrhagic fever. In addition, early appearance of DENV-specific T cell responses was significantly associated with milder clinical disease ( $p = 0.02$ ). DENV peptide specific T cell responses inversely correlated with the degree of viraemia, which was most significant for DENV-NS3 specific T cell responses (Spearman's  $r = -0.47$ ,  $p = 0.0003$ ). The frequency of NS1, NS5 and pooled DENV peptides, correlated with the degree of thrombocytopenia but had no association with liver transaminases. Our data suggest that early appearance of DENV-specific T cell IFN $\gamma$  responses appear to associate with milder clinical disease and resolution of viraemia, suggesting a protective role in acute dengue infection.

## Introduction

Dengue virus is the cause of the most common mosquito-borne viral infection worldwide, indeed over half of the global population live in areas where there is intense dengue transmission putting them at risk of dengue infection [1]. Dengue virus causes 390 million infections annually, of which nearly a quarter are clinically apparent causing a spectrum of disease phenotypes ranging from mild dengue fever (DF) to dengue hemorrhagic fever (DHF). DHF is defined by a transient increase in vascular permeability resulting in plasma leakage, with high fever, bleeding, thrombocytopenia and haemoconcentration, which can lead to shock (dengue shock syndrome (DSS))[2]. It is however not fully understood why some people develop more severe forms of the disease, with patient history, immunity, age, viral serotype, sub-strain and epidemiological factors all postulated to play a role [3]. It was highlighted during a recent summit to identify correlates of protection for dengue, that dengue virus (DENV) specific T cell immunity should be studied in more detail, in order to develop safe and effective dengue vaccines [4].

Although a dengue vaccine (Denvaxia) is now licensed in several countries, the efficacy is low in dengue seronegative individuals and provides only partial protection against DENV2 [5]. Although it is now generally believed that DENV specific T cells are protective, it is important that dengue vaccines should not induce “harmful” T cell immunity [4, 6–8]. Indeed, a significant hurdle in developing an efficacious dengue vaccine has been our limited understanding of the protective immune response in acute dengue infection and the added complexity of the presence of four DENV serotypes that are highly homologous. Although the evidence for T cells playing a possible protective role in DENV infections is emerging, there is still conflicting evidence as to the role of antigen-specific T cells during dengue infection is reported in the literature.

T cell responses to DENV are predominantly directed towards the nonstructural proteins (NS), with the majority of the CD8+ T cell responses directed towards NS3 followed by NS5 and CD4+ T cell responses to envelope, PrM and NS1 proteins [9–11]. It was believed that highly cross-reactive T cells specific to DENV-NS3, and other proteins, associate with severe clinical disease (DHF), and it was thought that these cells contribute to DHF by inducing a ‘cytokine storm’[12–15]. It is hypothesized in the ‘original antigenic sin’ theory that T cell

responses against the initial DENV serotype of primary infection persist and dominate during subsequent infections; and that these T cells are suboptimal in inducing robust antiviral responses upon re-challenge [13, 14, 16]. However, it has been shown that DENV-NS3 specific T cell responses were at very low frequency during acute disease, and only detected in the convalescent phase pointing away from a role in vascular leak [14, 16, 17]. Recently it was observed that DENV-specific T cells are found in large numbers in the skin during acute dengue infection, and it is speculated that highly cross-reactive, pathogenic skin T cells could be contributing to DHF, despite being absent or present at low frequencies in the peripheral blood [8, 18]. As the frequency of skin resident DENV-specific T cells was investigated in a small patient cohort, it is not yet clear whether the frequency of the skin T cells associated with clinical disease severity.

Conversely some studies in both humans and mouse models have shown that DENV-specific T cells in the blood are likely to be protective [19–23]. It was shown that in individuals who were naturally infected with DENV, polyfunctional CD8<sup>+</sup> T cells responses of higher magnitude and breadth were seen for HLA alleles associated with protection [21]. Similar findings were seen with DENV-specific CD4<sup>+</sup> T cell responses [23]. Our previous studies have also shown that the magnitude of IFN $\gamma$ -producing DENV NS3-specific memory T cell responses was similar in those who had varying severity of recovered past dengue infection, suggesting that the magnitude of the memory T cell response does not correlate with clinical disease severity [22]. While many studies have been carried out to elucidate the functionality of T cell responses in dengue, these have been limited to studying T cells specific for particular HLA types by using tetramers/pentamers [16, 18], or to investigating T cell responses in individuals with unknown severity of dengue.

To aid the generation of an effective vaccine it will be important to understand the role, phenotype and frequency of dengue-specific T cell responses in relation to clinical disease severity and clearance of viraemia [6, 7]. Therefore, here we investigate T cell responses to immunodominant DENV NS proteins in patients with DHF and DF, and analyse the association of such responses with resolution of viraemia.

## Methods

### Ethics statement

The study was approved by the Ethical Review Committee of The University of Sri Jayawardenepura. All patients were adults and recruited post written consent.

### Recruitment of patients for analysis of the functionality of T cell responses

We recruited 74 adult patients with acute dengue infection from the National Infectious Diseases Institute, between day 4–8 of illness, following informed written consent. The patients were followed up throughout their hospital stay and all clinical features were recorded several times each day, from time of admission to discharge. Ultra sound scans were performed to determine the presence of fluid leakage in pleural and peritoneal cavities. Full blood counts, and liver transaminase measurements were performed serially through the illness. Clinical disease severity was classified according to the 2011 WHO dengue diagnostic criteria [24]. Accordingly, patients with ultrasound scan evidence of plasma leakage (those who had pleural effusions or ascites) were classified as having DHF. Those who did not develop any clinical or laboratory features of plasma leakage throughout their hospital stay, were diagnosed as having DF. Shock was defined as having cold clammy skin, along with a narrowing of pulse pressure of  $\leq 20$  mmHg. Based on this classification, 45 patients had DHF and 29 patients had dengue fever (DF) of the 74 patients recruited for the study.

## Serology

Acute dengue infection was confirmed in serum samples using a PCR (see below) and dengue antibody detection. Dengue antibody assays were completed using a commercial capture-IgM and IgG ELISA (Panbio, Brisbane, Australia) [25, 26]. Based on the WHO criteria, those who had an IgM: IgG ratio of  $>1.2$  were considered to have a primary dengue infection, while patients with IgM: IgG ratios  $<1.2$  were categorized under secondary dengue infection [27]. The DENV-specific IgM and IgG ELISA was also used to semi-quantitatively determine the DENV-specific IgM and IgG titres, which were expressed in PanBio units.

## Qualitative and quantitative assessment of viral loads

DENV were serotyped and viral titres quantified as previously described [28]. RNA was extracted from the serum samples using QIAamp Viral RNA Mini Kit (Qiagen, USA) according to the manufacturer's protocol. Multiplex quantitative real-time PCR was performed as previously described using the CDC real time PCR assay for detection of the dengue virus [29], and modified to quantify the DENV. Oligonucleotide primers and a dual labeled probe for DENV 1,2,3,4 serotypes were used (Life technologies, India) based on published sequences [29]. In order to quantify viruses, standard curves of DENV serotypes were generated as previously described in Fernando, S. *et.al* [28].

## Peptides

The peptide arrays spanning DENV NS1 (DENV-1 Singapore/S275/1990, NS1 protein NR-2751), NS3 (DENV-3, Philippines/H87/1956, NS3 protein, NR-2754) and NS5 proteins (DENV-2, New Guinea C (NGC), NS5 protein, NR-2746) were obtained from the NIH Biodefense and Emerging Infections Research Resource Repository, NIAID, NIH. The DENV NS3 peptide array consisted of 105, 14–17 mers peptides, NS1 and NS5 proteins were comprised of 60 and 156 peptides respectively. The peptides were reconstituted as previously described [30]. NS1, NS3 and NS5 peptides were pooled separately to represent the DENV- NS1, NS3 and NS5 proteins. In addition, total NS1, NS3 and NS5 peptides were combined to represent a 'DENV-all' pool of peptides.

## Ex vivo ELISpot assay

Ex vivo IFN $\gamma$  ELISpot assays were carried out as previously discussed using freshly isolated peripheral blood mononuclear cells (PBMC) obtained from 74 patients [22]. Fresh PBMCs were used due to concerns of possible reduction/alterations in IFN $\gamma$  production by cryopreserved PBMCs [31]. DENV-NS3, NS1, NS5 and the combined DENV-ALL peptides were added at a final concentration of 10  $\mu$ M and incubated overnight as previously described [16, 32]. All peptides were tested in duplicate. PHA was included as a positive control of cytokine stimulation and media alone was applied to the PBMCs as a negative control. The spots were enumerated using an automated ELISpot reader (AID Germany). Background (PBMCs plus media alone) was subtracted and data expressed as number of spot-forming units (SFU) per 10<sup>6</sup> PBMCs.

## Quantitative cytokine assays

Quantitative ELISA for TNF $\alpha$  (Biolegend USA) and IL-2 (Mabtech, Sweden) were performed on ELISpot culture supernatants according to the manufacturer's instructions.

## Statistical analysis

PRISM version 6 was used for statistical analysis. As the data were not normally distributed, differences in means were compared using the Mann-Whitney U test (two tailed). Spearman rank order correlation coefficient was used to evaluate the correlation between variables including the association between DENV-specific T cell responses and platelet counts, degree of viraemia and liver transaminases.

## Results

### Patient clinical and laboratory features

To investigate the role of T cells in the progression of dengue infection we stratified patients based on disease severity. The clinical and laboratory features of the 74 patients recruited to the study are shown in Table 1. There was no statistically significant difference in the age of the individuals who had DF (median 29, IQR 29 to 42 years), when compared to those who had DHF (median 33, IQR 33 to 39 years). Of those who had DF, 18 (62.1%) were males and in those who had DHF 31 (68.9%) were males. Of these 74 patients, 45 had DHF and 29 had DF, and all 45 patients with DHF had ascites with 10 of them also experiencing pleural effusions. None of the patients developed shock and only one person progressed to bleeding manifestations (Table 1). The median duration of illness when recruited to the study was similar for patients with DF (median 5, IQR 5 to 6 days) and DHF (median 5, IQR 4 to 6 days).

**Table 1. Clinical and laboratory characteristics of patients with DHF and DF.**

Clinical findings	DHF (n = 45)	DF (n = 29)
Vomiting	15 (33.33%)	8 (27.59%)
Abdominal pain	25 (55.56%)	7 (24.14%)
Hepatomegaly	10 (22.22%)	2 (6.9%)
Bleeding manifestations	1 (2.22%)	0
Pleural effusion	10 (22.22%)	0
Ascites	45 (100%)	0
Lowest platelet count		
<20,000 cells/mm <sup>3</sup>	25 (55.56%)	1 (3.45%)
20,000 to 50,000	13 (28.89%)	10 (34.48%)
50,000–100,000	6 (13.33%)	12 (41.38%)
>100,000	1 (2.22%)	6 (20.69%)
Lowest Lymphocyte count		
<750	20 (44.44%)	7 (24.14%)
750–1500	20 (44.44%)	20 (68.97%)
>1500	5 (11.11%)	2 (6.90%)
Infecting serotype		
DENV1	14 (31.1)	16 (55.2)
DENV2	15 (33.3)	4 (13.8)
DENV3	3 (6.7)	1 (3.4)
DENV4	2 (4.4)	0 (0)
Aviraemia	11 (24.4)	8 (27.5)
Highest AST (IU/L)		
Median (IQR)	133.6 (86 to 366.1)	Median 72.5, IQR 55 to 183
Highest ALT (IU/L)		
Median (IQR)	172 (81 to 291)	90 (30.3 to 96)

<https://doi.org/10.1371/journal.pntd.0006540.t001>

### ***Ex vivo* IFN $\gamma$ responses in patients with acute dengue infection**

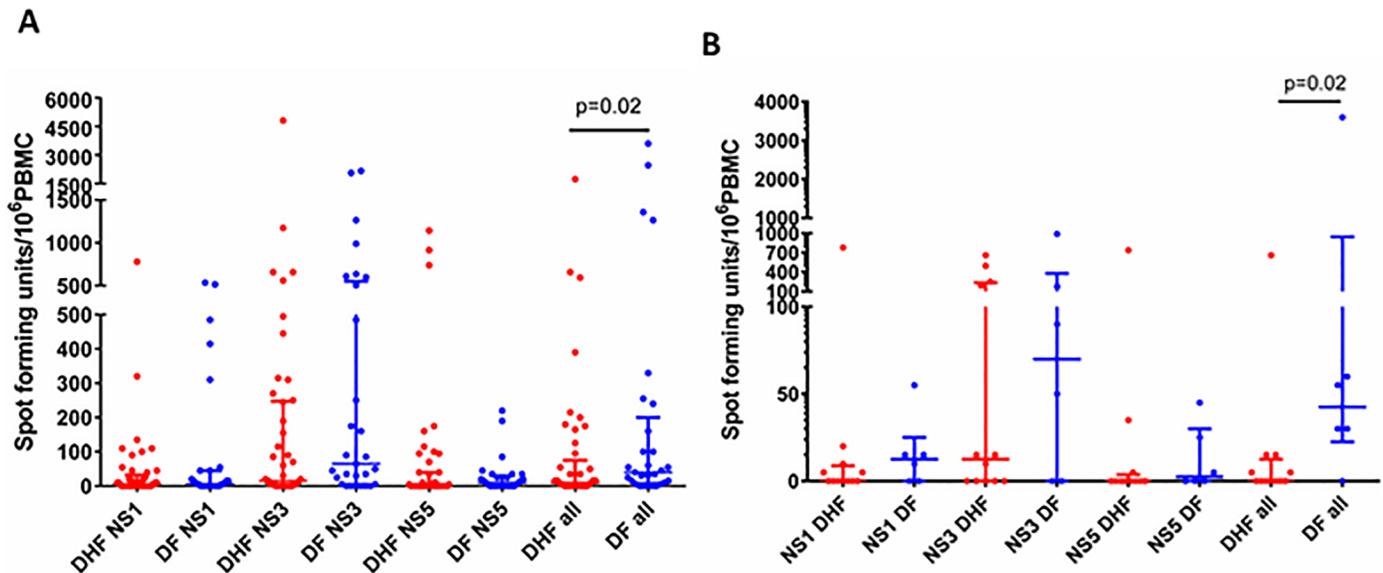
To evaluate the role of T cell derived cytokine in the immunopathology or regulation of acute dengue infection, we stimulated PBMCs isolated from patients with either DF or DHF with peptides constituting DENV-derived non-structural protein (NS) and assessed cytokine production by ELISPOT. We stimulated the patient PBMCs with different pools of overlapping peptides making up either full length NS1, NS3 or NS5 protein, or a pool of total NS1, 3 and 5 peptides (DENV-all). NS3 and NS5 were selected for investigation as CD8+ T cell responses have been shown to be directed to these proteins and CD4+ T cells have been shown to target structural proteins and NS1 as the main non-structural protein [11, 21]. This combination of NS proteins from the particular DENV strains has previously been used to study DENV specific T cell responses [33]. We used this *ex vivo* ELISPOT method to model antigen presentation of dengue-derived peptides to antigen-specific T cells in vitro and assessed T cell activation by IFN $\gamma$  production, as a representative cytokine produced by T cells during dengue infection. T cell responses to the pooled DENV peptides (DENV-ALL) ( $p = 0.02$ ) were higher in PBMCs derived from patients with DF than DHF patients and the NS3-specific responses showed a trend to be higher in those with DF than DHF (Fig 1A). T cell responses to DENV-NS1 peptides were similar in patients with DF and DHF. 26/45 patients with DHF and 12/29 patients with DF, had zero responses to NS5. We did not detect TNF $\alpha$  in the *ex vivo* ELISPOT culture supernatants, which is in contrast to studies performed by others on T cell clones that implied TNF $\alpha$  producing DENV-specific T cells contribute to disease pathogenesis [15]. We also did not detect significant quantities of IL-2. The T cell responses to the overlapping peptides were not different in males compared to females, and none of the peptide-specific responses correlated with the age of the individuals.

To assess if detection of DENV specific T cell responses before the onset of the critical phase (vascular leakage phase), was associated with a reduced likelihood of developing leakage, we isolated the data from DF and DHF patients recruited on day 4 post the onset of illness and analysed IFN $\gamma$  production by peptide stimulated PBMCs. None of the patients had evidence of vascular leakage on day 4 of illness and those who developed leakage (patients with DHF), did so on day 5 or 6. DF patients (those who did not develop any plasma leakage throughout their illness) had a significantly higher IFN $\gamma$  secretion response ( $p = 0.02$ ) to the DENV-all peptide pool (median 42.5, IQR = 22.5 to 945 SFU/10<sup>6</sup> PBMCs), when compared to DHF patients (median 0, IQR = 0 to 12.5 SFU/10<sup>6</sup> PBMCs) (Fig 1B). As such, significantly higher DENV-specific T cell responses were seen in those who did not develop fluid leakage, and those who had lower DENV-specific T cell responses proceeded to develop fluid leakage (DHF). Responses to DENV-NS3, NS1 and NS5 also appeared higher in patients with DF at this time point, although this did not reach statistical significance (Fig 1B).

To further assess the time-course of the response we obtained a second blood sample from eight patients within our cohort two days after collection of the first sample. T cell responses to DENV-ALL and DENV-NS3 peptides increased from the first sample (day 4) to the second (day 6), but it was not statistically significant ( $p > 0.05$ ) (S1 Fig)

### **Laboratory parameters and DENV-specific T cell responses**

Thrombocytopenia is associated with clinical disease severity and higher degrees of thrombocytopenia are seen in those with DHF compared to those with DF [24]. We found that DENV peptide specific T cell responses correlated with the degree of thrombocytopenia. While this correlation with T cell responses and platelet counts was significant for DENV-NS1 (Spearman's  $r = 0.26$ ,  $p = 0.01$ ) (Fig 2A), NS5 (Spearman's  $r = 0.4$ ,  $p = 0.0002$ ) (Fig 2B) and DENV-All (Spearman's  $r = 0.31$ ,  $p = 0.005$ ) (Fig 2C), it was not significant for NS3 (Spearman's  $r = 0.18$ ,



**Fig 1. Ex vivo ELISpot responses to DENV peptides in patients with DHF and DF.** (A) Ex vivo IFN $\gamma$  ELISpot responses to DENV NS1, NS3, NS5 and combined DENV overlapping peptides in patients with DF (n = 29) and DHF (n = 45). (B) Ex vivo IFN $\gamma$  ELISpot responses to DENV peptides in patients who were recruited on day 4 since onset of illness with DF (n = 6) and DHF (n = 12). Error bars represent the median and the interquartile range. \*P<0.05.

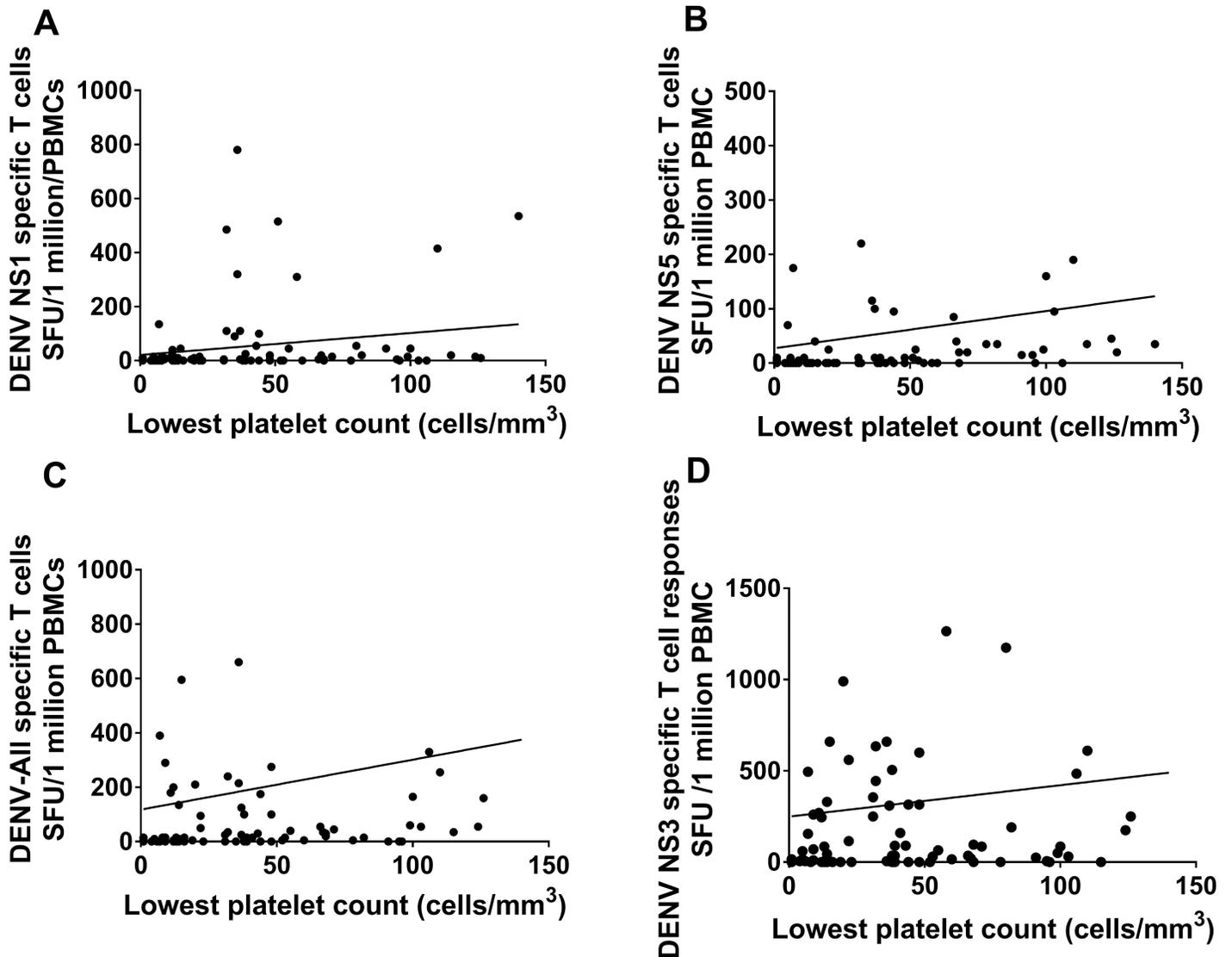
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p = 0.09) (Fig 2D). No association was seen with DENV-peptide specific T cell responses and aspartate transaminase (AST) and alanine transaminase (ALT) (S2 Fig), which are indicators of liver dysfunction [28, 34].

### The relationship between DENV serotype and T cell responses

While some studies report that certain DENV serotypes associate with DHF [35, 36], others have shown that the risk of DHF is similar regardless of serotype [37]. Therefore, we proceeded to determine whether there were differences in the T cell responses to DENV-proteins based on the viral serotype that the patients were infected with. Within our cohort 30 (40.5%) patients were infected with DENV1, 19 (25.7%) with DENV2, 4 (5.4%) with DENV-3 and 2 (2.7%) with DENV-4 (Table 1). The serotype could not be determined in 19 (25.7%) patients, as they were not viraemic at the time of recruitment. DHF developed in 14/30 (46.7%) of the patients infected with DENV-1 and 15/19 (78.9%) of those infected with the DENV-2 and in 11/19 (57.9%) who were aviraemic at the time of recruitment (Fig 3A). Thus, it appeared that DENV-2 infection was more likely to lead to development of DHF (odds ratio 3.3, 95% CI 0.93 to 12.1), however the association was not statistically significant (p = 0.08) in this cohort. Aviraemic individuals displayed significantly higher IFN $\gamma$  T cell responses to NS1 (p = 0.002), NS3 (p = 0.02), NS5 (p = 0.02) and DENV-ALL pooled peptides (p = 0.0004) when compared to those who were viraemic at the time of recruitment. In addition, those who were infected with the DENV-2 serotype, with a trend towards increased DHF susceptibility, had significantly lower responses to NS1 (p = 0.002), NS3 (p = 0.04), NS5 (p = 0.003) and DENV-All (p = 0.0003) peptides when compared to those who were infected with DENV-1.

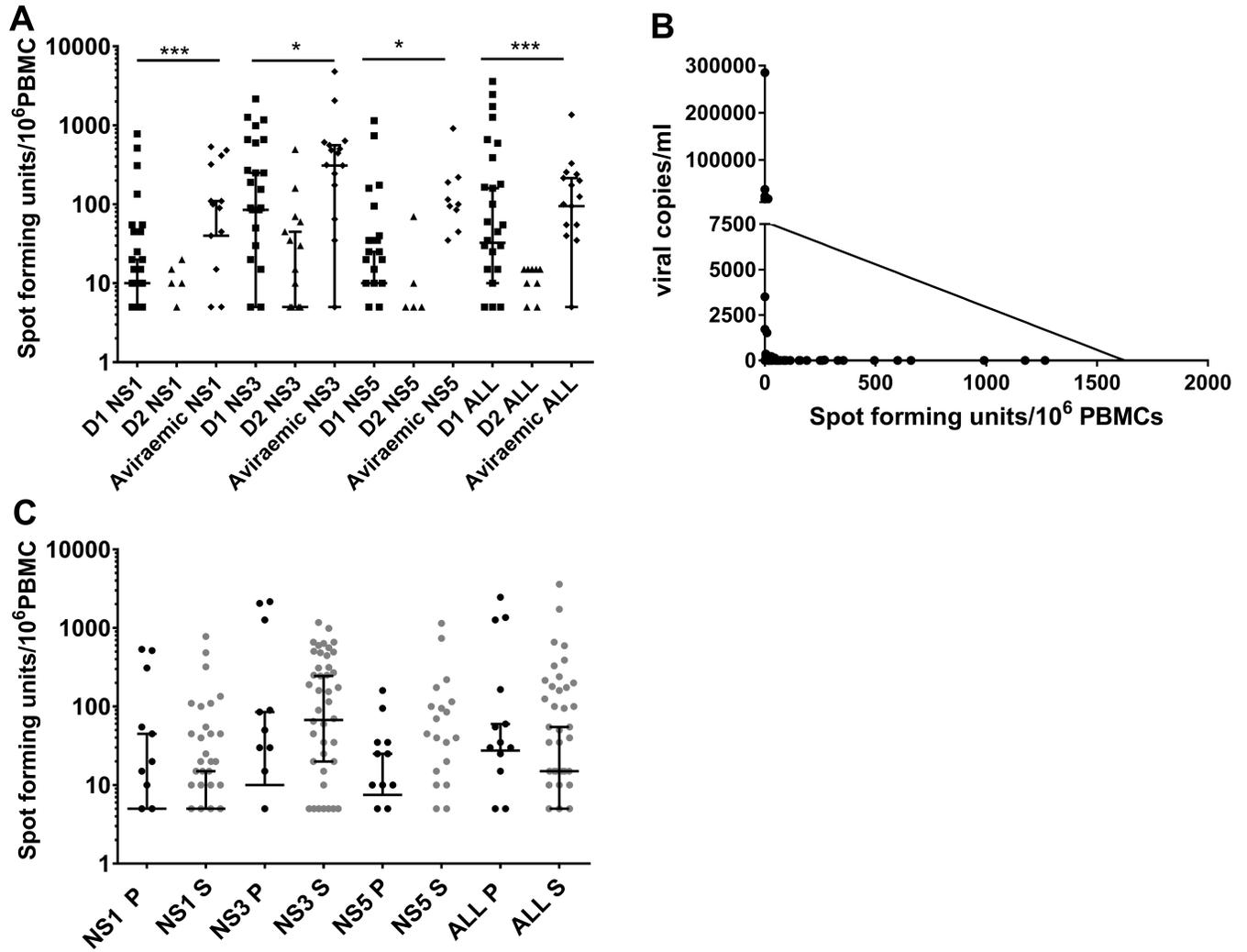
As the PBMCs of patients infected with different DENV serotypes, were stimulated with the non-structural proteins from different virus serotypes (NS1-DENV1, NS3-DEN3 and NS5-DEN2), we sought to assess the sequence identity of these proteins between serotypes. Multiple alignment of the NS5 protein sequences of DENV2 (58 sequences) and DENV3 (28 sequences) was performed using virus variation resource [38] and analysed using Clustal



**Fig 2. Relationship between laboratory parameters and DENV specific T cells in patients with acute dengue.** Platelet counts were correlated with ex vivo IFN $\gamma$  ELISpot responses to DENV NS1 (Spearman's  $r = 0.26$ ,  $p = 0.01$ ) (A), NS5 (Spearman's  $r = 0.4$ ,  $p = 0.0002$ ) (B) and the overall DENV (Spearman's  $r = 0.31$ ,  $p = 0.005$ ) (C) and NS3 (Spearman's  $r = 0.18$ ,  $p = 0.09$ ) (D) overlapping peptides in patients ( $n = 74$ ).

<https://doi.org/10.1371/journal.pntd.0006540.g002>

omega and showed a sequence identity of  $> 72.1\%$  between the NS5 proteins of these viral serotypes [39]. Multiple alignment of the NS3 protein of DENV2 (61 sequences) and DENV3 (28 sequences) showed a sequence identity of  $> 72.02\%$ . Multiple alignment of the NS1 protein of DENV2 (62 sequences) and DENV3 (28 sequences) showed a sequence identity of  $> 65.11\%$ . The homology between DENV1 and DENV2 NS5 was  $> 71.83\%$  (comparison of 102 DENV1 sequences and 58 DENV2 sequences) [38], while the homology between DENV1 and DENV3 NS3 was  $> 76.9\%$  (comparison of 102 DENV1 sequences and 28 DENV3 sequences) [38]. Therefore, the T cell responses to different DENV serotypes is unlikely to be profoundly influenced by using non-structural protein sequences of different DENV serotypes. However, since there was some concern that stimulating using different serotypes would influence the results, we carried out further analysis. Since the most immunodominant protein was NS3, which was from DENV3, we also compared NS3 specific T cell responses in patients with



**Fig 3. Relationship between DENV serotype, clinical disease severity, viraemia and T cell responses.** A: Ex vivo ELISpot responses to DENV NS1, NS3, NS5 and combined DENV-ALL overlapping peptides in patients who were infected with DENV-1 (n = 30), DENV-2 (n = 19) or who were aviraemic (n = 19). Error bars represent the median and the interquartile range. \*\*P<0.05, \*\*\*P<0.01, \*\*\*\*P<0.001. (B) Correlation between DENV NS3-specific T cell responses and degree of viraemia (Spearman's  $r = -0.47$ ,  $p = 0.0003$ ). (C) Ex vivo ELISpot responses to DENV NS1, NS3, NS5 and the overall DENV overlapping peptides in patients who had primary dengue (P) (n = 19) and secondary dengue infection (S) (n = 48).

<https://doi.org/10.1371/journal.pntd.0006540.g003>

DENV1 (n = 30) and DENV2 (n = 19). Although we did not find any significant difference in the T cell responses to NS3 based on infecting DENV serotype ( $p = 0.06$ ), the responses to DENV3 NS3, appeared higher in patients with an acute DENV1 infection (median 85, IQR 5 to 435 SFU/million PBMBs) compared to those with an acute DENV2 infection (median 10, IQR 0 to 66.25 SFU/million PBMBs) (S3A Fig).

We then went on to compare responses to NS5 peptides, which were derived from DENV2, in patients with an acute DENV1 and DENV2 infection. Again, those with an acute DENV2 infection had significantly lower responses to the NS5 ( $p < 0.0001$ ) compared to patients with DENV1 (S3B Fig). We believe this difference is due to 15/19 (78.9%) patients with DENV2 having DHF (and thus poor T cell responses), whereas only 14/30 (46.7%) of those with DENV1 had DHF. We carried out a similar comparison within the group of patients with acute DENV1 for NS1 (which was from a DENV1 strain) and again did not see any difference

within the DENV1 group in those with DF and DHF (S3C Fig), which was compatible with responses of the overall results.

### Viraemia and DENV specific T cell responses

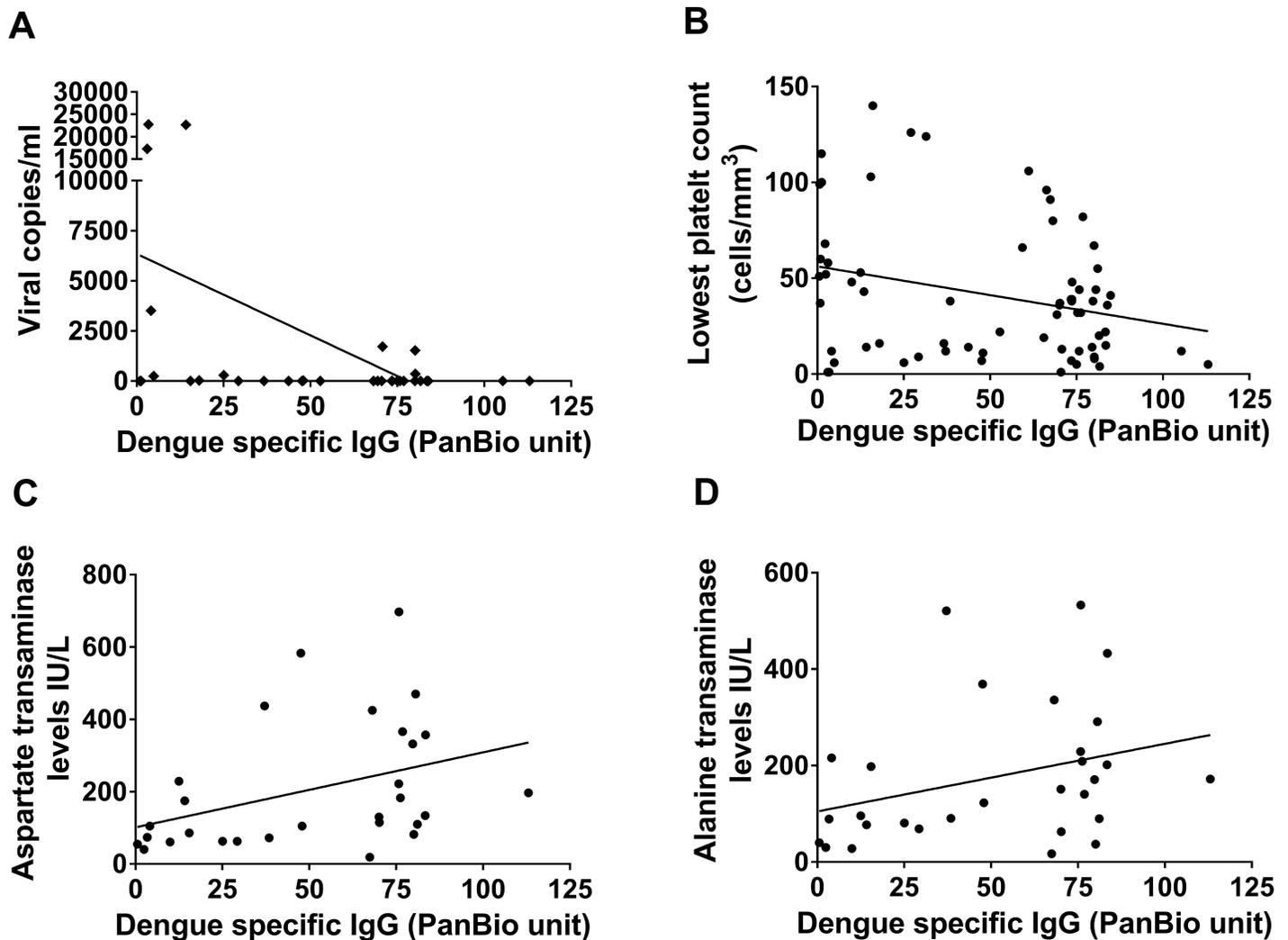
DHF patients have been shown to have higher viral loads, exhibit prolonged viraemia [40, 41] and persistent DENV-NS1 antigenaemia [42, 43]. As such we attempted to elucidate a correlation between T cell cytokine responses and viremia. DENV specific T cell responses to NS1, NS3 and NS5 peptides in addition to the pooled peptides (DENV-ALL) inversely correlated with the degree of viraemia, which was most significant for DENV-NS3 specific T cell responses (Spearman's  $r = -0.47$ ,  $p = 0.0003$ ) (Fig 3B and S4 Fig). The viral loads significantly inversely correlated with the platelet counts (Spearman's  $r = -0.34$ ,  $p = 0.01$ ), with the platelet counts being lowest in individuals with the highest viral loads.

It is thought that a second dengue virus infection with a different viral serotype is a risk factor for developing DHF[44]. To determine the effect of secondary infection on the resulting T cell response, we characterized patient infection history and assayed patient blood for the presence of dengue specific IgM and IgG. Primary infection was defined by DENV-specific IgM: IgG >1.2 [24]. Accordingly, 19 (25.7%) patients were classified as experiencing a primary dengue infection and 48 (64.9%) were defined as secondary dengue infection. The antibody results were inconclusive for 7 (9.4%) patients. Our results showed no significant difference in DENV specific T cell responses between primary and secondary dengue infection patient groups ( $p > 0.05$ ) for any of the DENV peptide pools (Fig 3C).

We semi-quantitatively determined the DENV-specific IgM and IgG antibody titres in all patients with DF and DHF, and we found that neither the DENV-IgM nor IgG antibody titres correlated with T cell responses to DENV-NS1, NS5 and NS3. However, the DENV-specific IgG antibody titres inversely correlated with viral loads in those with DHF (Spearman's  $r = -0.37$ ,  $p = 0.03$ ) (Fig 4A), but not in those with DF (Spearman's  $r = -0.25$ ,  $p = 0.16$ ). In analysis of the IgG antibody titres of all patients ( $n = 74$ ) they too inversely correlated with the degree of thrombocytopenia (Spearman's  $r = -0.29$ ,  $p = 0.009$ ) (Fig 4B). DENV-specific IgG also correlated with the highest aspartate transaminase (AST) (Spearman's  $r = 0.51$ ,  $p = 0.004$ ) (Fig 4C) and alanine transaminase (ALT) levels (Spearman's  $r = 0.4$ ,  $p = 0.03$ ) in all patients with acute dengue infection (Fig 4D).

### Discussion

In this study we set out to investigate the role of T cells in dengue immunity and found that DENV-specific T cells are present at low frequency during acute infection, consistent with previous reports published by us and by others [16, 17, 45]. IFN $\gamma$  production was significantly higher in patients with DF as opposed to DHF, especially during early infection. Those who had lower DENV-NS3 specific T cell responses on day four since the onset of illness (before development of fluid leakage), were significantly more likely to subsequently develop vascular leakage of DHF. In addition, the frequency of pooled DENV-peptide specific, in particular DENV-NS3 specific, T cell responses was associated with resolution of viraemia. Aviraemic patients had significantly higher DENV-specific T cell responses when compared to those who were viraemic. However, overall the majority of patients with both DF and DHF, had a very low magnitude of DENV specific T cell responses. T cell IFN $\gamma$  responses to DENV NS1, NS5 and pooled DENV (NS1, NS3 and NS5) peptides inversely correlated with the degree of thrombocytopenia, but we did not show any relationship with liver transaminases (AST and ALT levels). Both the degree of thrombocytopenia and a rise in both AST and ALT, have been shown to associate with dengue severity [24, 28]. Therefore, our data show that the early appearance (on day 4 of illness



**Fig 4. DENV-specific IgG responses and laboratory parameters of clinical disease severity in patients with acute dengue.** Analysis of the correlation between DENV-specific IgG responses and (A) degree of viraemia (Spearman's  $r = -0.25$ ,  $p = 0.16$ ) in patients with DHF, (B) degree of thrombocytopenia (Spearman's  $r = -0.29$ ,  $p = 0.009$ ), (C) aspartate transaminase (AST) levels (Spearman's  $r = 0.51$ ,  $p = 0.004$ ) and (D) alanine transaminase (ALT) levels (Spearman's  $r = 0.51$ ,  $p = 0.004$ ) in all patients ( $n = 74$ ) with acute dengue infection.

<https://doi.org/10.1371/journal.pntd.0006540.g004>

before any patient developed plasma leakage) of DENV-NS3 specific T cell responses is associated with milder disease, which is compatible with recent studies regarding the role of T cells in DENV infection [18, 21–23]. This suggests that DENV-peptide specific T cells are possibly have a protective role against developing severe forms of dengue infection.

Although Appana et al also evaluated *ex vivo*  $IFN\gamma$  to selected peptides of structural and non-structural DENV proteins by ELISpot assays, they did not find any differences in the frequency of DENV-specific T cell responses in patients with DF when compared to those with DHF [46]. However, only peptides that were predicted to bind to certain major HLA alleles were included in the authors' peptide pools used in the ELISpot assays [46], whereas here we utilised peptides spanning the entire length of DENV NS1, NS3 and NS5, proteins. This difference in experimental approach may have affected the cytokine production profile of the responding T cells in the different disease states. In addition, as the viability and function of T cells have been shown to be affected in those with acute dengue infection [31], we used freshly

isolated PBMCs in all our experiments to limit extraneous cellular stress in contrast to previous studies [15, 21, 46, 47].

In this study, some of the non-structural peptides used were of a different DENV serotype than that causing the acute infection as we used fresh PBMCs in our assays before the infecting serotype was known. As many previous studies on T cell responses have used peptides of unmatched DENV serotypes to assess T cell responses [33, 46] and also as it was shown that the breadth or the magnitude of DENV specific T cell responses, were similar even when peptides of unmatched serotypes were used [9], we too proceeded with the same approach. However, there is a possibility that using unmatched peptides could affect the magnitude of the T cell responses. Subsequent sub-analysis of T cell responses to DENV1-NS1 and DENV3-NS3 of patients with an acute DENV1 and DENV2 infection, carried out by us also did not show any difference. However, analysis of T cell responses to DENV2 derived NS5 overlapping peptides, showed that patients with an acute DENV2 infection (matched peptides) had significantly lower responses than patients with DENV1 infection. We believe this difference is possibly due to 78.9% patients with DENV2 having DHF (and thus poor T cell responses), whereas only 46.7% of those with DENV1 had DHF.

As with most viral infections, the severity of the illness and T cell responses to the DENV has also been shown to depend on the HLA type of the individual [21, 23, 48]. However, we did not assess the T cell responses in relation to their HLA type as the primary aim of this study was to assess the overall DENV specific T cell responses based on clinical disease severity and viraemia, irrespective of their HLA types in a large cohort, with relevance to pathogenesis and vaccine design. As the HLA type is known to influence the magnitude of the T cell response, there is a possibility that the differences in the magnitude of T cell responses to different DENV overlapping peptides, could also be due to the differences in their HLA types, which were not assessed here.

In general, more severe forms of dengue infection are observed during a secondary heterologous dengue infection [4], which gave rise to the hypothesis that cross reactive T cells responding to the primary infecting DENV serotype are suboptimal in clearing the secondary virus, and lead to development of more severe disease [14, 16]. In these studies, it was shown that a tetramer of different viral specificity to the current infecting DENV serotype, sometimes had a higher affinity to the DENV specific T cells [16]. In our study, we did not observe any difference in IFN $\gamma$  production in overall *ex vivo* ELISpot assays from PBMCs derived from patients with primary or secondary dengue infection; however, we did not examine variant peptide-specific responses. The broad differences we observed in DENV-specific T cell responses correlated only with clinical disease severity. Interestingly, the DENV-specific IgG levels, which were measured semi-quantitatively, inversely correlated with the degree of thrombocytopenia and also AST and ALT levels, which are known to associate with liver damage. DENV-specific IgG levels are known to be significantly higher in patients with secondary dengue, compared to primary dengue, indeed it is one of the criteria for definition of a secondary dengue infection. However, as we assessed the relationship of total IgG with these laboratory parameters and did not assess the presence of neutralizing antibodies, which are more likely to be protective, this needs to be further evaluated.

In summary, we found that DENV-specific T cell IFN $\gamma$  responses, were associated with milder clinical disease severity and resolution of viraemia, suggesting a protective role for peptide specific T cells early in acute dengue infection.

## Supporting information

**S1 Fig. Frequency of DENV specific NS1, NS3, NS5 and All overlapping peptide responses in patients with acute dengue (n = 8) on day 4 and day 6 of illness.**

(TIF)

**S2 Fig.** Association of DENV specific NS1, NS3, NS5 and All overlapping peptide responses in patients with acute dengue (n = 74) with the highest recorded aspartate transaminase level (NS1 Spearman's  $r = 0.15$ ,  $p = 0.4$ ; NS3 Spearman's  $r = 0.34$ ,  $p = 0.05$ ; NS5 Spearman's  $r = 0.16$ ,  $p = 0.37$ ; All Spearman's  $r = 0.33$ ,  $p = 0.06$ ) (A) and alanine transaminase level (NS1 Spearman's  $r = 0.12$ ,  $p = 0.5$ ; NS3 Spearman's  $r = 0.25$ ,  $p = 0.15$ ; NS5 Spearman's  $r = 0.19$ ,  $p = 0.29$ ; All Spearman's  $r = 0.31$ ,  $p = 0.08$ ) (B). (TIF)

**S3 Fig. T cell responses of patients infected with DENV1 and DENV2 for different non structural proteins.** (A) IFN $\gamma$  ELISpot responses were assessed in patients with acute DENV1 (n = 30) and acute DENV2 (n = 19) to NS3 overlapping peptides derived from DENV3 (B) IFN $\gamma$  ELISpot responses were assessed in patients with acute DENV1 (n = 30) and acute DENV2 (n = 19) to NS5 overlapping peptides derived from DENV2 (C) IFN $\gamma$  ELISpot responses were assessed in patients with acute DENV1 resulting in DF (n = 16) or DHF (n = 14) for NS1 overlapping peptides derived from DENV1. (TIF)

**S4 Fig. Relationship between viraemia and DENV T cell responses.** (A) Correlation between DENV All-Specific T cell responses and degree of viraemia (Spearman's  $r = -0.38$ ,  $p = 0.004$ ). (B) Correlation between DENV NS5-Specific T cell responses and degree of viraemia (Spearman's  $r = -0.28$ ,  $p = 0.04$ ). (C) Correlation between DENV NS1-Specific T cell responses and degree of viraemia (Spearman's  $r = -0.31$ ,  $p = 0.02$ ). (TIF)

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