

## ORIGINAL RESEARCH

# Association of dengue virus-specific polyfunctional T-cell responses with clinical disease severity in acute dengue infection

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## Funding information

Medical Research Council (UK); National Institute for Health Research (NIHR) Oxford Biomedical Research Centre (BRC); Centre for Dengue Research, University of Sri Jayawardenapura; National Science Foundation, Sri Lanka, Grant/Award Number: (NSF/RG/2016/HS/05)

## Abstract

**Introduction:** Although the role of dengue virus (DENV)-specific T cells in the pathogenesis of acute dengue infection is emerging, the functionality of virus-specific T cells associated with milder clinical disease has not been well studied. We sought to investigate how the functionality of DENV-NS3 and DENV-NS5 protein-specific T cells differ in patients with dengue fever (DF) and dengue hemorrhagic fever (DHF). **Methods:** Using intracellular cytokine assays, we assessed the production of interferon  $\gamma$  (IFN $\gamma$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), macrophage inflammatory protein-1 $\beta$  (MIP-1 $\beta$ ), and CD107a expression in adult patients with acute DF (n = 21) and DHF (n = 22).

**Results:** Quadruple cytokine-producing, polyfunctional DENV-NS3- and DENV-NS5-specific T cells were more frequent in those with DF when compared to those with DHF. While DENV-NS3- and DENV-NS5-specific T cells in patients with DF expressed IFN $\gamma$  > TNF- $\alpha$  > MIP-1 $\beta$  > CD107a, T cells of those with DHF predominantly expressed CD107a > MIP-1 $\beta$  > IFN $\gamma$  > TNF- $\alpha$ . Overall production of IFN $\gamma$  or TNF- $\alpha$  by DENV-NS3- and DENV-NS5-specific T cells was significantly higher in patients with DF. The majority of NS3-specific T cells in patients with DF (78.6%) and DHF (68.9%) were single-cytokine producers; 76.6% of DENV-NS5-specific T cells in those with DF and 77.1% of those with DHF, produced only a single cytokine. However, no significant association was found with polyfunctional T-cell responses and the degree of viraemia.

**Conclusions:** Our results suggest that the functional phenotype of DENV-specific T cells are likely to associate with clinical disease severity.

## KEYWORDS

clinical disease severity, cytokines, dengue, polyfunctional T cells

## 1 | INTRODUCTION

Dengue viral infections affect approximately 390 million individuals annually, of which 96 million infections manifest as apparent dengue infections.<sup>1</sup> Those who have an apparent dengue infection have symptoms ranging from mild dengue fever (DF) to dengue hemorrhagic fever (DHF). DHF is the severe form of disease which is characterized by plasma leakage resulting in pleural effusions, ascites, and shock along with risk of thrombocytopenia, bleeding, and organ dysfunction.<sup>2</sup> Currently, there is no specific treatment for dengue and careful monitoring of patients for development of complications and fluid management is the only management strategy available. The only licensed dengue vaccine was found to have low efficacy for some serotypes and has been cautioned for the ability to cause severe disease in dengue-naïve individuals.<sup>3,4</sup> This is despite this vaccine inducing high levels of neutralizing antibodies<sup>5</sup> and dengue virus (DENV)-specific T-cell responses of similar breadth and magnitude to those seen following natural infection.<sup>6</sup> Therefore, it appears that that we need to further understand the constituents of a protective immune responses for development of safer and effective dengue vaccines.

Although there is still some controversy regarding the role of T cells in the pathogenesis of dengue infection, more recent studies have shown that DENV-specific T cells are likely to have a protective role.<sup>7-10</sup> Recently, we reported that patients with DF had significantly higher DENV-specific T-cell responses than those with more severe forms of illness, which appeared very early in illness.<sup>11</sup> Moreover, we also found that the magnitude of the DENV-specific T-cell response inversely correlated with the degree of viraemia, further suggesting that T cells have a protective role in acute dengue.<sup>11</sup>

Apart from the breadth and magnitude of a T-cell response, their functionality has also been shown to be crucial in protection against many infections.<sup>12-14</sup> Polyfunctional T cells that produce multiple types of cytokines have been shown to be protective and correlate with resolution of viraemia, especially in chronic viral infections.<sup>15-17</sup> Polyfunctional T-cell responses have shown to correlate with the degree of protection with vaccines, rather than pathogen-specific T cells inducing interferon  $\gamma$  (IFN $\gamma$ ) responses alone.<sup>18</sup> Polyfunctional virus-specific T cells have also shown to be important in prevention of virus reactivation in infections such as cytomegalovirus.<sup>12</sup>

It was shown that in individuals who were naturally infected with the DENV, polyfunctional CD8+ and CD4+ T-cell responses of higher magnitude and breadth were seen for the human leukocyte antigen (HLA) alleles

associated with protection.<sup>7,9</sup> We observed that although individuals who were naturally infected with the DENV resulting in either inapparent or a severe dengue infection had the same magnitude of DENV-specific T-cell responses, they differed in functionality. For instance, while DENV-specific T cells of those who had previous inapparent dengue made higher levels of granzyme B, those with past severe disease made more TNF- $\alpha$ .<sup>19</sup> Therefore, as with many other infections, apart from the magnitude and breadth of the T-cell response, the type of cytokines produced by virus-specific T cells appear to associate with protection or pathogenesis. While many studies have been carried out to elucidate the functionality of T-cell responses in dengue, these have been limited to studying T-cell specific for particular HLA types by using tetramers/pentamers.<sup>10,20</sup> As the functionality of DENV-specific T cells in acute dengue have not been assessed previously in relation to clinical disease severity and resolution of viraemia, we sought to determine whether polyfunctional CD8+ T-cell responses to DENV were associated with milder disease and early resolution of viraemia.

## 2 | METHODS

### 2.1 | Recruitment of patients

We recruited 43 adult patients with acute dengue infection, during a single outbreak from the National Infectious Diseases Institute following informed written consent. Patients were recruited between days 3 and 7 of illness and all clinical features were recorded several times per day, from time of admission to discharge. Ultrasound scans were carried out to identify the presence of fluid leakage in pleural and peritoneal cavities. Full-blood counts and liver-transaminase levels were performed serially during the duration of illness. Clinical disease severity was classified as per the 2011 WHO dengue diagnostic criteria.<sup>2</sup> Accordingly, patients with pleural effusions or ascites or those with a rise in their hematocrits 20 mm Hg or higher of their baseline were classified as having DHF. Shock was defined as the presence of cold clammy skin, in combination with a narrowing pulse pressure of 20 mm Hg or lower. On the basis of this classification, 22 patients had DHF and 21 patients had DF.

### 2.2 | Ethics statement

The study was approved by the Ethical Review Committee of The University of Sri Jayewardenepura. All patients were recruited following written consent.

## 2.3 | Serology

Acute dengue infection was confirmed in serum samples by polymerase chain reaction (PCR) and dengue antibody identification. Dengue antibody assays were performed using a commercial capture-immunoglobulin M (IgM) and IgG ELISA (Panbio, Brisbane, Australia).<sup>21</sup> According to WHO criteria, patients with an IgM:IgG ratio of more than 1.2 were considered under primary dengue infection, while patients with IgM:IgG ratios less than 1.2 were categorized under secondary dengue infection.<sup>2</sup> The DENV-specific IgM and IgG enzyme-linked immunosorbent assay (ELISA) was also performed to determine the semiquantitative DENV-specific IgM and IgG titers and the results were reported in PanBio units.

## 2.4 | Qualitative and quantitative assessment of viral loads

DENV viruses serotyping and quantification was carried out as previously described.<sup>22</sup> RNA was extracted from the serum samples using QIAamp Viral RNA Mini Kit (Qiagen) as per the manufacturer's protocol. Multiplex quantitative real-time PCR was performed as previously reported using the CDC real-time PCR assay to detect DENV. Furthermore, the technique was modified to quantify the DENV. Oligonucleotide primers, a dual-labeled probe for all DENV 1 to 4 serotypes (Life Technologies, India) were used and this was based on published sequences.<sup>23</sup> To quantify viruses, standard curves of DENV serotypes were produced as previously reported in Fernando et al.<sup>22</sup>

## 2.5 | Peptides

The peptide arrays spanning NS3 (DENV3, Philippines/H87/1956, NS3 protein, NR-2754) and NS5 proteins (DENV2, New Guinea C, NS5 protein, NR-2746) from the NIH Biodefense and Emerging Infections Research Resource Repository, NIAID, NIH were used in the study. DENV-NS3 peptide array is comprised of 105, 14- to 17-mer peptides while NS5 proteins were consisted of 156 peptides. The peptides were reconstituted as previously reported.<sup>24</sup> DENV-NS3 and -NS5 peptides were pooled separately to represent the DENV-NS3 and -NS5 proteins. Final concentration of dimethyl sulfoxide is less than 0.5%.

## 2.6 | Intracellular cytokine assays

The following monoclonal antibodies from Biologend were used in this study after optimization by serial dilutions: anti-CD3-APC Cy7 (clone OKT3),

anti-CD8-BV650 (clone SK1), anti-IFN- $\gamma$ -APC (clone 4S. B3), anti-CD107a-FITC (clone H4A3), anti-TNF- $\alpha$ -BV605 (clone Mab11). Anti-MIP1- $\beta$ -PE (clone 24006) was purchased from R&D Systems and LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, for 405 nm excitation was from Thermo Fisher Scientific. Intracellular staining was carried out as previously described.<sup>25,26</sup> Briefly, to determine CD107a expression, peripheral blood mononuclear cells (PBMCs) were stained with anti-CD107a-fluorescein isothiocyanate (FITC) monoclonal antibodies for 30 minutes at 1 to 2  $\times 10^6$ /mL in RPMI 1640 plus 10% fetal calf serum (FCS), before stimulation with the antigen. The PBMCs were then stimulated with either DENV-NS3 or DENV-NS5 15-mer overlapping peptides for 16 hours according to manufacturer's instructions in the presence of Brefeldin A and Monensin (Biologend) as previously described.<sup>25,27</sup> The PBMCs were stained with anti-CD3 and -CD8, permeabilized and fixed with Cytotfix/Cytoperm (Biologend) and then stained for intracellular TNF- $\alpha$ , IFN $\gamma$ , or MIP-1 $\beta$  as previously described.<sup>25,26</sup> LIVE/DEAD Fixable Aqua Dead Cell Stain was used according to the manufacturer's protocol to exclude dead cells.

Samples were acquired using a Guava-easy Cyte 12 HT Flowcytometer (Merck) and analyzed with FCS Express 6 Flow Research Edition. A hierarchical gating strategy was used to gate live, single, CD3+, and CD8+ T cells (Figures S1 and S2). Each antibody was titrated to determine the optimum concentration to use by comparing it with Fluorescence Minus One (FMO) controls. Gates for TNF- $\alpha$ , MIP1- $\beta$ , IFN- $\gamma$ , and CD107a production in DENV-NS3- and DENV-NS5-stimulated cells were set using the unstimulated controls (Figure S2). After the gates for each effector function were created, the data were analyzed using Boolean combination gating to test 15 response patterns for the four functions tested as previously described.<sup>28</sup> Unstimulated controls were used in all assays.

## 2.7 | Statistical analysis

Statistical analysis was carried out using PRISM version 6. As the data were not normally distributed, differences in means were compared using the Mann-Whitney *U* test (two-tailed). The Spearman rank-order correlation coefficient was used to evaluate the correlation between variables. The differences in the frequency the various combinations of single, double, and triple cytokine-producing DENV-specific T cells in patients with DF and DHF were analyzed using the Holm-Sidak method. The relative proportion of single, double, triple, and quadruple cytokine-producing DENV-NS3- and DENV-NS5-specific T cells in patients with DF and

DHF, was calculated as previously described.<sup>28</sup> The differences between the single, double, triple, and quadruple cytokine-producing T cells in patients with DF and DHF was analyzed using the Mann-Whitney *U* test (two-tailed).

### 3 | RESULTS

#### 3.1 | Clinical and laboratory characteristics of patients

The clinical and laboratory features of the patients are shown in Table 1. In summary, 22 of 43 patients had DHF and 21 had DF. Nineteen of 22 (86.4%) DHF patients displayed ascites and 10 of 22 (45.4%) had pleural effusions. None of the patients developed shock and only one of 43 patients developed bleeding manifestations. The median duration of illness when recruited to the study for patients with DF was 5, (IQR, 4-5) days and for patients with DHF was 6 (IQR, 5-7) days.

#### 3.2 | Frequency of production of different cytokines by DENV-specific T-cell responses in acute dengue

During an acute secondary dengue infection, the quality and quantity of memory T-cell responses to the previously infecting DENV are likely to shape the immune responses in subsequent dengue infections. To assess the functionality of the DENV-specific T-cell response in relation to clinical disease severity, we initially assessed their ability to produce different cytokines. The responses to DENV-NS3 and -NS5 pool of 15-mer peptides were investigated as T-cell responses in acute dengue, as these have shown to be the two main proteins inducing CD8+ T-cell responses.<sup>7,29,30</sup>

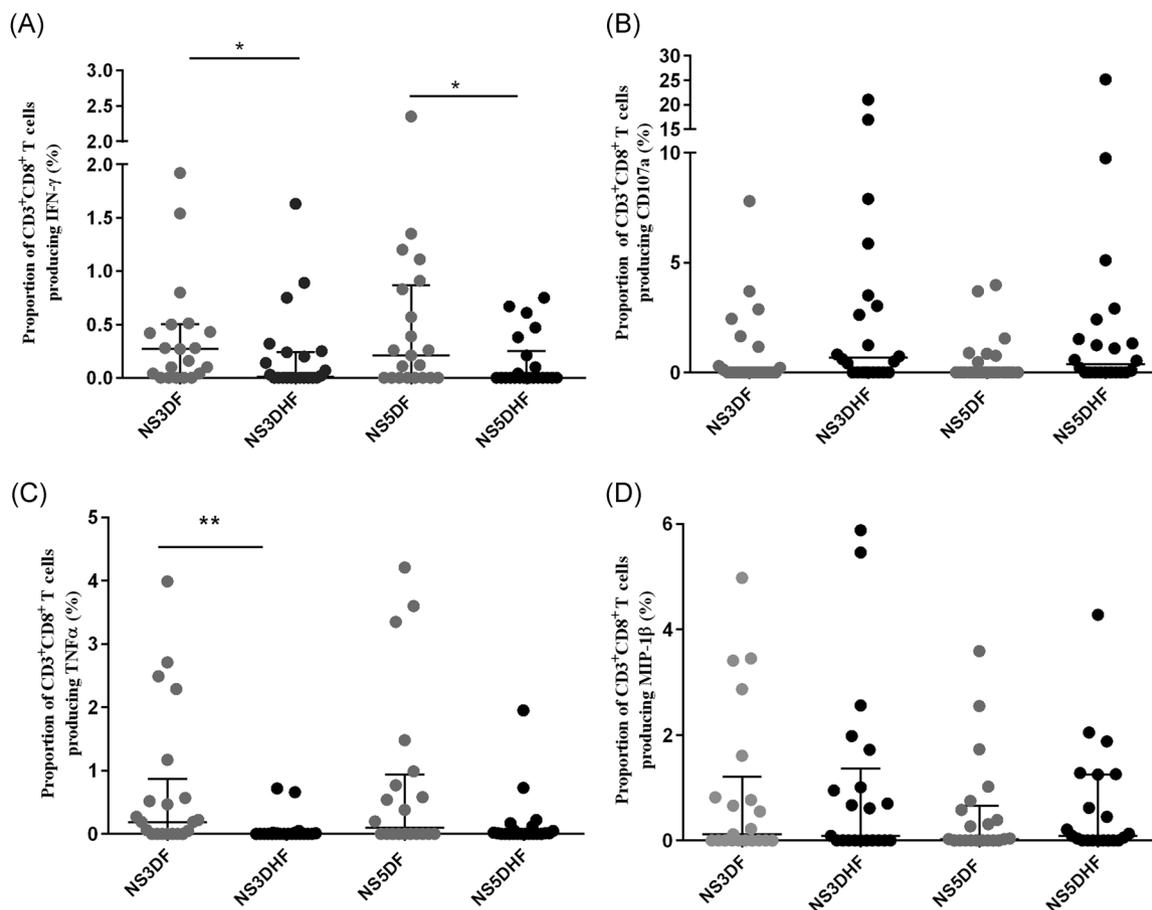
DENV-NS3-specific CD8+ T cells of patients with DF produced significantly more ( $P = .045$ ) IFN $\gamma$  (median = 0.28; IQR, 0%-0.51%) compared to those of patients with DHF (median, 0; IQR, 0%-0.24%) (Figure 1A). DENV-NS5-specific CD8+ T cells of patients with DF also produced significantly more ( $P = .046$ ) IFN $\gamma$  (median, 0.21; IQR, 0%-0.87%) compared to those with DHF (median, 0; IQR 0%-0.28%) (Figure 1A). Although the frequency of TNF- $\alpha$  producing DENV-NS3-specific CD8+ T cells were significantly higher ( $P = .003$ ) in patients with DF (median, 0.2; IQR, 0%-0.87%) compared to patients with DHF (median, 0; IQR, 0%-0%) (Figure 1C) there was no significant difference seen for DENV-NS5-specific CD8+ T cells between DF and DHF patients ( $P = .17$ ). In contrast, although not significant, CD107a expression was higher in patients with DHF for both DENV-NS3 (median, 0.61; IQR,

0%-3.5% of CD8+ T cells) and DENV-NS5-specific T-cell responses (median, 0.32; IQR, 0%-1.48% of CD8+ T cells) compared to patients with DF who exhibited lower CD107a expression following DENV-NS3 (median, 0; IQR, 0%-1.14% of CD8+ T cells) and DENV-NS5 stimulation (median, 0; IQR, 0%-0.69% of CD8+ T cells) (Figure 1B). Ten of 21 (47.6%) of those with DF and 13 of 22 (59.1%) of those with DHF upregulated CD107a in response to NS3. Only two patients with DF had expression rates of greater than 3% whereas seven patients with DHF had rates over 3%. When stimulated with NS5, 7 of 21 (33.3%) of those with DF expressed CD107a compared to 13 of 22 (59.1%) of those with DHF. However, there did not appear to be a difference in MIP-1 $\beta$  production in patients with DHF for both DENV-NS3 (median, 0.09; IQR, 0%-1.3% of CD8+ T cells) and -NS5 (median, 0.09; IQR, 0%-1.25% of CD8+ T cells) compared to those with DF (for NS3, median, 0.11; IQR, 0%-1.23% and for NS5, median, 0; IQR, 0%-0.65% of CD8+ T cells) (Figure 1D).

**TABLE 1** Clinical and laboratory features of patients with DF and DHF

	DHF (n = 22)	DF (n = 21)
Clinical findings	N (%)	N (%)
Vomiting	5 (22.7)	5 (23.8)
Abdominal pain	12 (54.5)	9 (42.9)
Hepatomegaly	4 (18.2)	0
Bleeding manifestations	1 (4.5)	0
Pleural effusion	10 (45.5)	0
Ascites	19 (86.4)	0
Lowest platelet count, cells/mm <sup>3</sup>		
<20 000	11 (52.4)	0
20 000-50 000	7 (33.3)	1 (4.8)
50 000-100 000	2 (9.5)	9 (42.9)
>100 000	1 (4.5)	11 (52.4)
Lowest lymphocyte count		
<750	2 (9.5)	3 (14.3)
750-1500	8 (38.1)	10 (47.6)
>1500	11 (52.4)	8 (38.1)
Infecting serotype		
DENV1	0	0
DENV2	19 (90.5)	16 (76.2)
DENV3	0	0
DENV4	0	0
Aviraemia	2 (9.5)	5 (23.8)
Inconclusive	0	1 (4.7)

Abbreviations: DENV, dengue virus; DF, dengue fever; DHF, dengue hemorrhagic fever.



**FIGURE 1** Overall production of cytokines by DENV-NS3- and DENV-NS5-specific T cells in patients with DF and DHF. IFN $\gamma$  (A), CD107a (B), TNF- $\alpha$  (C), and MIP-1 $\beta$  (D) was measured by surface and intracellular cytokine staining in patients with DF (n = 21) and DHF (n = 22). The error bars indicate the median and the IQR. DENV, dengue virus; DF, dengue fever; DHF, dengue hemorrhagic fever; IFN $\gamma$ , interferon  $\gamma$ ; IQR, interquartile range; MIP-1 $\beta$ , macrophage inflammatory protein-1 $\beta$ ; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ . \* $P$  < .05 and \*\* $P$  < .001

On the basis of the IgM and IgG ratios, 35 of 43 patients could be classified as having either primary or secondary dengue, based on the WHO guidelines.<sup>2</sup> Of the 35 patients, 17 had a primary dengue infection and 18 had a secondary dengue infection. There was no difference in production of cytokines (IFN $\gamma$ , TNF- $\alpha$ , and MIP-1 $\beta$ ) in those with primary dengue compared to secondary dengue, when stimulated with either NS3 or NS5. However, although not significant, there was a trend towards increase in CD107a expression in patients with secondary dengue compared to those with primary dengue for both NS3 and NS5.

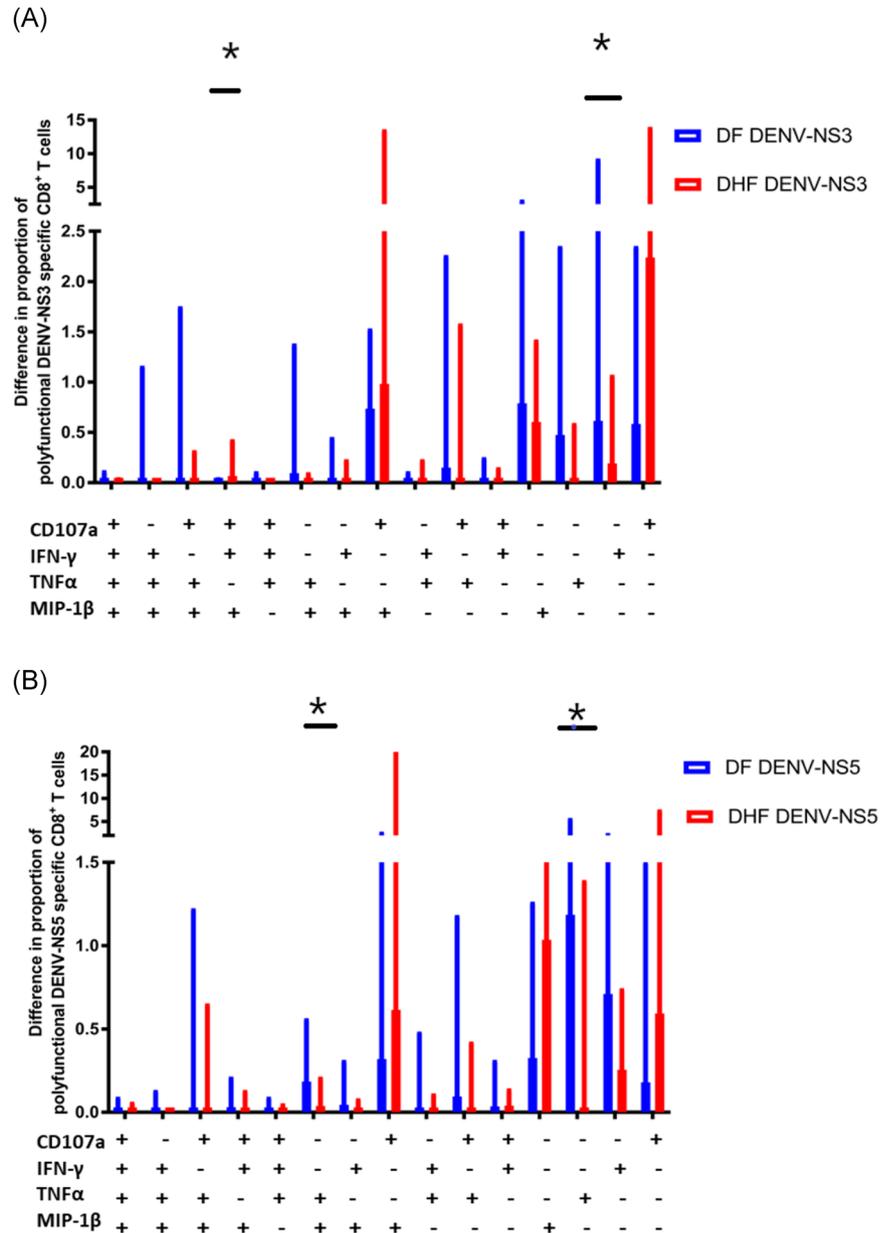
### 3.3 | Association of DENV-specific polyfunctional CD8+ T cells and disease severity

To assess the polyfunctionality of DENV-responsive T cells we assessed the ability of T cells to produce multiple cytokines or their ability to carry out certain effector

functions by determining the frequency of 15 different combinations of IFN $\gamma$ , TNF- $\alpha$ , CD107a, and MIP-1 $\beta$  production by DENV-NS3- (Figure 2A) and DENV-NS5 (Figure 2B)-specific CD8+ T cells in patients with DF and DHF. To correct for multiple corrections, the data were analyzed using the Holm-Sidak method. While DENV-NS3-specific T cells that produced a combination of CD107a/IFN $\gamma$ /MIP-1 $\beta$  were significantly higher in patients with DHF ( $P$  = .049), DENV-NS3-specific T cells that produced TNF- $\alpha$  alone was significantly higher ( $P$  = .02) in patients with DF (Figure 2A). DENV-NS5-specific T cells which produced a combination of TNF- $\alpha$ /MIP-1 $\beta$  and those which produced TNF- $\alpha$  alone were significantly higher in those with DF ( $P$  = .03 and  $P$  = .02, respectively; Figure 2B).

Overall, in patients with DF, DENV-NS3-specific T cells producing IFN $\gamma$  alone (median, 0.21; 0%-0.61% of CD8+ T cells) were more frequent than those producing TNF- $\alpha$  (median, 0.1; IQR, 0%-0.45% of CD8+ T cells). The frequency of DENV-NS3-specific T cells in patients with DF were as

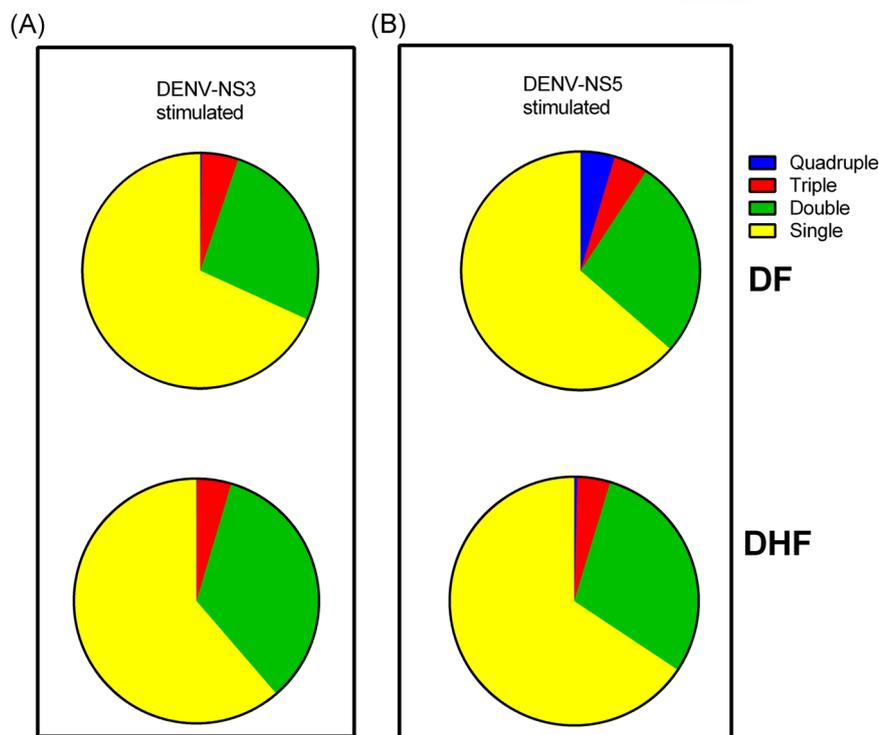
**FIGURE 2** Frequency of single, double, triple, and quadruple cytokine-producing DENV-NS3- and DENV-NS5-specific T cells in patients with DF and DHF. Fifteen combinations of cytokine-producing T cells were assessed by intracellular cytokine staining in patients with DF (n = 21) and DHF (n = 22) by using the Boolean combination gates. The error bars indicate the median and the IQR. DENV, dengue virus; DF, dengue fever; DHF, dengue hemorrhagic fever; IFN $\gamma$ , interferon  $\gamma$ ; IQR, interquartile range; MIP-1 $\beta$ , macrophage inflammatory protein-1 $\beta$ ; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ . \* $P < .05$



follows: IFN $\gamma$  > MIP-1 $\beta$  > TNF- $\alpha$  > CD107a > CD107a/MIP-1 $\beta$  > CD107a/TNF- $\alpha$  > CD107a/TNF- $\alpha$ /MIP-1 $\beta$  producing cells. The frequency DENV-NS3-specific T cells in patients with DHF were as follows: CD107a > CD107a/MIP-1 $\beta$  > MIP-1 $\beta$  > IFN $\gamma$ . The frequency of DENV-NS5-specific T cells in patients with DF were as follows: TNF- $\alpha$  > IFN $\gamma$  > CD107a/MIP-1 $\beta$  > MIP-1 $\beta$  > CD107a > TNF- $\alpha$ /MIP-1 $\beta$ . The frequency of DENV-NS5-specific T cells in patients with DHF were as follows: CD107a/MIP-1 $\beta$  > CD107a > IFN $\gamma$  > MIP-1 $\beta$ . Therefore, the cytokine signature of DENV-NS3- and DENV-NS5-specific T cells appear to be very different in those with DF compared to those with DHF, with the DENV-NS3- and DENV-NS5-specific T cells predominantly expressing CD107a and MIP-1 $\beta$  and those with DF predominantly producing IFN $\gamma$  and TNF- $\alpha$ .

### 3.4 | The association between number of cytokine-producing T cells and clinical disease severity

Next, we compared quality of the DENV-specific T-cell responses in patients with DF and DHF by calculating the frequency of single, double, triple, and quadruple effector DENV-NS3- and DENV-NS5-specific CD8<sup>+</sup> T-cell proportions regardless of cytokine/effector function combination within the DENV-specific cytokine-producing CD8<sup>+</sup> T-cell population (Figure 3). We did not find any significant differences between single, double, triple, and quadruple DENV-NS3- and DENV-NS5-specific CD8<sup>+</sup> T-cell proportions within the DENV-specific cytokine-producing CD8<sup>+</sup> T-cell population in patients with DF and DHF ( $P > .05$ ).



**FIGURE 3** Proportions of polyfunctional T cells in patients with DF and DHF. The proportion of single, double, triple, and quadruple cytokine-producing (A) DENV-NS3- and (B) NS5-specific T cells were compared in patients with DF ( $n = 21$ ) and DHF ( $n = 22$ ). DENV, dengue virus; DF, dengue fever; DHF, dengue hemorrhagic fever

0.2% NS3-specific T cells in patients with DF were quadruple cytokine/effector producers ( $\text{IFN}\gamma$ ,  $\text{TNF-}\alpha$ ,  $\text{CD107a}$ , and  $\text{MIP1}\beta$ ) while only 0.04% of DENV-NS3-specific T cells in patients with DHF expressed all four effectors. Furthermore 4.6% of DENV-NS5-specific T cells were quadruple cytokine/effector producers in patients with DF and only 0.4% in patients with DHF. Therefore, DENV-NS3- and NS5-specific T cells of those with DF were more likely to be polyfunctional. The majority of NS3-specific T cells in patients with DF (78.6%) and DHF (68.9%) were single cytokine/effector producers. A similar trend was seen for responses to NS5, with 76.6% of those with DF and 77.1% of those with DHF, producing only a single cytokine/effector. Only 21.4% of NS3-specific T cells in patients with DF and 23.4% in those with DHF produced two cytokines/ effectors and for NS5, only 22.7% of those with DF and 18.1% of those with DHF, produced two.

### 3.5 | Relationship between functionality of DENV-specific T cells and viraemia

In our previous study, we found that  $\text{IFN}\gamma$ -producing DENV-NS3-specific T cells inversely associated with the degree of viraemia.<sup>11</sup> However, in this study, we did not observe such a relationship with  $\text{IFN}\gamma$ , but instead found that the overall DENV-NS5-specific  $\text{MIP-1}\beta$  producing  $\text{CD8}^+$  T cells negatively correlated with the degree of viraemia in patients with DF (Spearman  $r = -0.51$ ,

$P = .018$ ), but not in those with DHF. We further analyzed the relationship between polyfunctional T-cell responses and the degree of viraemia. We only found that in patients with DHF, NS5-specific T cells expressing a combination of  $\text{CD107a}/\text{MIP-1}\beta/\text{IFN}\gamma$ , positively and significantly correlated with the degree of viraemia (Spearman  $r = 0.44$ ,  $P = .046$ ). No other cytokine combination had any association with the degree of viraemia.

## 4 | DISCUSSION

In this study, we have shown that quadruple cytokine/effector-expressing, polyfunctional DENV-NS3- and DENV-NS5-specific T cells were more frequent in those with DF when compared to those with DHF. In addition, the hierarchy of cytokine production was significantly different in those with severe and milder forms of dengue. For instance, while in those with DF, DENV-NS3-, and NS5-specific T cells expressed  $\text{IFN}\gamma > \text{TNF-}\alpha > \text{MIP-}\beta > \text{CD107a}$ , those with DHF were of predominantly  $\text{CD107a} > \text{MIP-}\beta > \text{IFN}\gamma > \text{TNF-}\alpha$ . Overall production of  $\text{IFN}\gamma$  or  $\text{TNF-}\alpha$  by DENV-NS3- and DENV-NS5-specific T cells was significantly higher in patients with DF. Therefore, the preferential production of  $\text{TNF-}\alpha$  and  $\text{IFN}\gamma$ , which are two potent antiviral cytokines by  $\text{CD8}^+$  T cells, compared with its degranulation capacity appeared to associate with milder clinical disease. However, in this study, we only investigated the functionality of  $\text{CD8}^+$  T-cell responses in patients with

acute DENV2 infection, as this was the only circulating serotype during this time period in Sri Lanka.<sup>31</sup> Although in our previous study, we did not observe any differences in the frequency of IFN $\gamma$  producing DENV-specific T cells in acute DENV1 and DENV2 infection, it would be important to study the polyfunctionality of the DENV-specific T-cell responses during infections with different DENV serotypes.

Although the overall frequency of DENV-NS3- and DENV-NS5-specific T cells was low for all cytokines that were evaluated, they were comparable to the frequencies of antigen-specific T cells seen in the acute phase of other virus infections such as influenza,<sup>32</sup> measles,<sup>33</sup> and Ebola.<sup>34</sup> Therefore, although we observed a low frequency of DENV-specific CD8+ T-cell responses, these lower frequencies are likely to be important in controlling the viraemia, as seen with acute viral infections. In our previous studies, we have shown that those with milder forms of dengue have higher frequencies of DENV-specific T-cell responses,<sup>11,25</sup> whereas, the majority of patients with severe disease did not have any detectable virus-specific T-cell responses. In this study, DENV-specific T cells produced significantly higher IFN $\gamma$  and TNF- $\alpha$  in those with milder disease, further suggesting that the DENV-specific T cells appear to associate with milder disease.

There has been some speculation that proinflammatory cytokines produced by DENV-specific T cells contribute to clinical disease severity by leading to endothelial dysfunction.<sup>20,35,36</sup> Highly cross-reactive, DENV-specific CD8+ T-cell clones, which produced cytokines such as TNF- $\alpha$ , were thought to contribute to vascular leak. Indeed, anti-TNF- $\alpha$  treatment in dengue mouse models was shown to increase survival in experimental DENV infection.<sup>37</sup> However, it has been shown that DENV-specific T-cell responses were absent or present in very low frequencies when the patient enters the critical phase of illness, and that TNF- $\alpha$  production by T cells did not associate with severe disease.<sup>20,25,38,39</sup> Indeed it was shown that children who had a higher frequency of DENV-specific T cells producing TNF- $\alpha$ , IFN $\gamma$ , and interleukin-2 were more likely to have the development of subclinical infection rather than symptomatic illness.<sup>40</sup> In human and animal influenza viral infection, TNF- $\alpha$  was shown to have more potent antiviral effects than IFN $\gamma$  or IFN $\alpha$ .<sup>41</sup> Therefore, both TNF- $\alpha$  and IFN $\gamma$  production by DENV-specific T cells appear to be important in reducing disease severity, rather than causing disease pathogenesis. However, neither TNF- $\alpha$  or IFN $\gamma$  production by CD8+ T cells correlated with the degree of viraemia. Instead, it was MIP-1 $\beta$  producing CD8+ T cells in patients with DF, which inversely correlated with the degree of viraemia. Although we previously reported that IFN $\gamma$ -producing

T cells as determined by ex vivo ELISpot inversely correlated with the viraemia, we believe that we did not see a similar observation, as we only investigated CD8+ T cells in this study compared with the overall DENV-T-cell responses in our previous study.<sup>11</sup>

In healthy individuals who were naturally exposed to the DENV, the majority of DENV-specific T cells produced two cytokines, irrespective whether they were simulated with conserved- or serotype-specific peptides.<sup>7</sup> However, we found that in acute infection only 18.1% to 23.3% of DENV-specific T cells produced two cytokines/ effectors, possibly due to the downregulation of T-cell receptor signaling pathways involved in cytokine during acute dengue.<sup>38</sup> Although the mean frequency of single cytokine-/effector-producing T cells was between 0.1% and 1.9% of the CD8+ T-cell population, the mean frequency of T cells producing a combination of TNF- $\alpha$ /IFN $\gamma$ , TNF- $\alpha$ /MIP-1 $\beta$ , or IFN $\gamma$ /MIP-1 $\beta$  was less than 0.3% of all CD8+ T cells. However, CD107a/MIP-1 $\beta$  expressing CD8+ T cells in patients with DHF was between 1.2% and 1.5% of all CD8+ T cells. The expression of CD107a on the surface of CD8+ T cells has shown to be a reliable marker of cytotoxic ability of these cells.<sup>42</sup> Although expression of CD107a by CD8+ T cells is thought to associate with protection,<sup>43</sup> it has shown to cause disease pathogenesis in some infections.<sup>44</sup> For instance, in cutaneous Leishmaniasis, CD107a expressing cytotoxic T cells were highly active in lesions and in mouse models, it was shown that perforin-deficient mice, did not develop skin lesions when compared with wild-type mice.<sup>44</sup> Furthermore, it was shown that disease progression was not associated with the degree of parasitaemia, but rather with the extent of the presence of cytotoxic T cells in skin lesions.<sup>44</sup> However, although we found that CD107a expressing or CD107a/MIP-1 $\beta$ /IFN $\gamma$  expressing DENV-specific T cells were higher in patients with DHF, there was no association with the degree of viraemia. Therefore, it appears that CD107a expression might not always be protective and DENV-specific T cells that are preferentially cytotoxic, as opposed to those producing IFN $\gamma$  and TNF- $\alpha$ , associate with disease pathogenesis.

In this study, we used overlapping peptides of the NS3-DENV3 to assess NS3 responses in patients with an acute DENV2 infection. Although there was some concern that using peptides from different serotypes may induce different responses to other infecting serotypes, due to the similarity in the sequences, many previous studies have used a similar approach.<sup>20,27,45</sup> In addition, it was also shown that HLA-A\*11-NS3 epitope-specific tetramer responses (specific for DENV2) were similar in patients with an acute DENV1 or DENV2 infection.<sup>20</sup>

In summary, we investigated polyfunctional DENV-NS3- and DENV-NS5-specific T-cell responses

in patients with acute dengue and found that DENV-specific T cells producing quadruple cytokines/effectors were more frequent in patients with DF, and they had a different functional phenotype. While DENV-specific T cells in those with milder disease preferentially produced  $IFN\gamma > TNF-\alpha > MIP-1\beta > CD107a$ , those with severe disease had a functional phenotype of  $CD107a > MIP-1\beta > IFN\gamma > TNF-\alpha$ . Therefore, our results suggest that not only the magnitude of T-cell responses, but also the functional phenotype of DENV-specific T cells associate with disease severity.

## ACKNOWLEDGMENTS

Funding was provided by the Centre for Dengue Research, University of Sri Jayewardenapura, 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).

## CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

## DATA AVAILABILITY

All data is shown within the manuscript, figures, and Supporting Information.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** Wijeratne DT, Fernando S, Gomes L, et al. Association of dengue virus-specific polyfunctional T-cell responses with clinical disease severity in acute dengue infection. *Immun Inflamm Dis.* 2019;7:276-285. <https://doi.org/10.1002/iid3.271>