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Immune activation is a feature of dengue hemorrhagic fever (DHF) and CD8+ T cell responses in particular have been suggested as having a role in the vasculopathy that characterizes this disease. By phenotyping CD8+ T cells (CD8+/HLA-DR+, CD8+/Ki-67+, or HLA-DR+/Ki-67+) in serial blood samples from children with dengue, we found no evidence of increased CD8+ T cell activation prior to the commencement of resolution of viremia or hemoconcentration. Investigations with MHC class I tetramers to detect NS3133–142-specific CD8+ T cells in two independent cohorts of children suggested the commencement of hemoconcentration and thrombocytopenia in DHF patients generally begins before the appearance of measurable frequencies of NS3133–142-specific CD8+ T cells. The temporal mismatch between the appearance of measurable surface activated or NS3133–142-specific CD8+ T cells suggests that these cells are sequestered at sites of infection, have phenotypes not detected by our approach, or that other mechanisms independent of CD8+ T cells are responsible for early triggering of capillary leakage in children with DHF. The Journal of Immunology, 2010, 184: 7281–7287.

Dengue is an acute mosquito-borne disease that is widespread in tropical and subtropical countries. Dengue hemorrhagic fever (DHF) is a serious form of dengue that is characterized by a vasculopathy accompanied by altered hemostasis. The loss of water and small molecules from the intravascular space into surrounding tissues are the physiological hallmarks of the vasculopathy in DHF and this process begins in the first few days of illness (1).

The pathogenesis of dengue is likely multifactorial. Viral traits as well as host genetic and physiological factors may each contribute to the clinical phenotype (2–4). In children and adults, epidemiological evidence suggests that two sequential dengue virus (DENV) infections, with the second infection caused by a different DENV serotype to the first, is associated with elevated risk (but is not an absolute requirement) for DF (5–8). Infants born to dengue immune mothers can also develop DHF as a result of primary infection (9, 10). The most prominent hypothesis to explain both epidemiological observations is that non-neutralizing or subneutralizing levels of anti-DENV Abs (acquired by previous infection or passively in utero) can enhance DENV infection in Fc receptor-bearing cells; a hypothesis that is supported by in vitro (11–14) and some in vivo (animal model) evidence (11, 15). The mechanisms through which Ab-dependent enhancement of DENV infection might result in the characteristic vasculopathy seen in DHF patients is not fully defined.

Ag-driven immune activation during secondary infection may be a crucial mechanistic link between high viral burdens and the vasculopathy of DHF. In particular, the rapid mobilization of memory serotype cross-reactive memory T cells that release vasodilatory inflammatory molecules has been suggested to be important in secondary infection (16, 17). Consistent with this, circulating plasma levels of soluble CD8 and T cell-derived molecules, such as IFN-γ and sIL-2R, are higher in DHF patients than dengue fever (DF) patients at the time of defervescence, albeit not corrected for the hemoconcentration present in DHF patients (18). In addition, a higher frequency of surface activated CD8+CD69+ T cells are present in DHF cases during the febrile phase than in patients with milder disease (19).

Data on DENV-epitope–specific responses during the febrile phase is sparse. We previously showed that DENV-epitope–specific responses are difficult to detect in the acute febrile phase by ELISPOT assay (20). Similarly, CD8+ T cells specific for the dominant HLA-A*11–restricted epitope NS3133–142 were present only at very low frequency during the febrile phase in Thai children, but were readily detected in early convalescence (21). Other studies to characterize DENV-epitope–specific T cell responses have used PBMCs collected in late convalescence and so it is difficult to know whether these findings can be extrapolated to the febrile phase (22, 23).

A systematic description of the kinetics of CD8+ T cell activation, and particularly DENV-specific CD8+ T cells, in the context of dynamic changes in viremia and hemoconcentration would provide further insights into the role of these cells in immunity and pathogenesis. To this end, the aim of the current study was to measure the timing of specific and nonspecific CD8+ T cell responses and virological and clinically important hematological events during the febrile phase of dengue.
Materials and Methods

Patient recruitment

Two prospective studies are described. In the first study, consecutive children (<15 y) with suspected dengue and <72 h of illness were enrolled at a private clinic in Ho Chi Minh City, Vietnam, between October 2007 and March 2008. All patients were examined by the same physician (Dr. Tran Van Ngoc). Blood samples were collected daily during the febrile period for measurement of viremia and routine hematological investigations. T cell phenotyping and tetramer staining was performed every second day. Convalescent blood samples were obtained 2–3 wk after first presentation. The extent of hematocrit in dengue cases was determined by comparing the maximum hematocrit recorded during the acute phase with the baseline hematocrit. Baseline hematocrit was defined as the lowest of either the value recorded before day 2 of illness if platelet count was ≥200,000 (available for 60% of patients) or the value recorded at follow-up 2–3 wk after illness onset (available for 82% of cases), or a mean of age- and sex-matched healthy population value (13% of cases).

The second study was a prospective study of children with dengue at Pediatric Hospital no. 1 and no. 2 between August 2006 and March 2007. In patients >6 mo of age with suspected dengue and fever for <7 d were enrolled in the study. All patients were assessed daily by a study physician and had daily serial hematological measurements and an ultrasound within 24 h of defervescence. In study 2, the extent of hematocrit was determined by comparing the daily maximum hematocrit recorded during hospitalization with a mean age- and sex-matched healthy population baseline hematocrit.

The platelet nadir in studies 1 and 2 was defined as the lowest platelet count recorded in patients with a minimum of four platelet counts. The day of defervescence was defined as the day the fever dropped below <37.5˚C (axillary) and remained so for 48 h. For calibration purposes, the day of defervescence was defined as “fever day (FD) 0,” with the day prior to defervescence defined as “FD −1.” The day of illness was as self-reported by the patient’s parent or guardian. For both studies, if patients received laboratory confirmation of dengue, then World Health Organization clinical classification criteria (24) were applied to each case. Written informed consent was obtained from a parent or guardian of each study participant. The study protocols were approved by the Scientific and Ethical committees at each participating hospital.

Dengue diagnostics

A capture IgM and IgG ELISA assay (Venture Technologies, Sarawak, Malaysia) that used inactivated viral Ag from DENV1–4 was performed as described previously (25). DENV levels in plasma were measured using an internally controlled, serotype-specific, real-time RT-PCR assay that has been described previously (26). Results were expressed as cDNA equivalents per milliliter of plasma. A diagnosis of confirmed dengue was made if the PCR was positive and/or if there was evidence of rising IgM titers to DENV Ag in paired plasma samples and the response to DENV Ag was greater than to JEV Ag. The interpretation of primary and secondary serological responses was based on the magnitude of IgG ELISA units in early and convalescent serum samples taking into account the day of illness. The cutoff in IgG ELISA units for distinguishing primary from secondary dengue by day of illness was calibrated using a panel of acute and early convalescent sera from Vietnamese dengue patients that were assayed at the Centre for Vaccine Development, Mahidol University (Bangkok, Thailand), using a reference IgM and IgG Ag capture ELISA described previously (27).

HLA typing

HLA typing for HLA-A*11 alleles was performed by PCR-specific sequence primers as previously described (28). Each PCR reaction contained a unique set of allele-specific PCR primers (amplifying alleles HLA-A*1101/02/03/04/05/06/07/08/09/11/12/13 alleles), and a set of internal control primers (amplifying a conserved region from the third intron of the HLA-DRB1 locus). The presence of an allele was defined by the presence of the allele-specific PCR product and negative when only the internal control product is present.

Flow cytometry and tetramers

Flow cytometric analysis of whole-blood stained with fluorochrome-conjugated monoclonal Abs was performed on a FACS Canto II flow cytometer (Becton Dickinson, San Diego, CA). Cell surface staining was routinely performed on 100 μl fresh whole blood. All Abs were purchased from Becton Dickinson. HLA-A*1101 MHC class I tetramers containing the NS3_133–142 epitope from all four DENV serotypes were synthesized as previously described (21). A comparison of the peptide epitopes used in tetramer folding versus the NS3_133–142 epitope present in 815 DENV genotypes sampled from dengue patients in southern Vietnam between 2001 and 2008 indicated there were no mismatches between the selected peptides and the NS3_133–142 epitope sequences found in DENV-1 (590/590 [100%]) or DENV-3 (31/31 [100%]) genome sequences. For DENV-2, there was only one DENV-2 variant of the NS3_133–142 epitope in these Vietnamese sequences that is not represented in our tetramer pool. This variant was present at a very low frequency (1/194 sequences, 0.5%). For DENV-4, no Vietnamese sequences were available for analysis. The four tetramers (DV1NS3_133–142 GTSGSPINVR, DV2.1NS3_133–142 GTSGSPIDDK, DV2.2NS3_133–142 GTSGSPIVDK, and DV3-4NS3_133–142 GTSGSPINHR) were pooled at equal concentrations and used to stain fresh whole blood. Briefly, whole blood was stained with pooled PE-labeled HLA-A*1101 tetramers at 37˚C for 30 min in dark and then APC-conjugated anti-CD8 mAb was added for 15 min at 37˚C. Stained blood was then lysed, washed, fixed, and analyzed by flow cytometry. The limit of detection was set at three times the SD of the mean percentage of tetramer positive events in 23 patients with no virological or serological evidence of acute dengue. We verified that the tetramers were capable of binding the relevant TCR by staining a cross-reactive CD8 T cell clone generated from a Vietnamese patient with dengue and described previously (29).

Statistical analysis

The nonparametric Mann-Whitney U test was used to evaluate differences in continuous data. Two sides p values <0.05 were considered statistically significant. The program Prism (version 5.0) was used for analyses (GraphPad Software, San Diego, CA).

Results

Characteristics of study population

The characteristics of the 126 patients in whom CD3+CD8+ T cells were phenotyped are described in Table I. These patients were a subset of 138 consecutively enrolled patients at an outpatient primary care clinic of whom there were 12 patients who were not investigated because of inadequate samples (clotted or insufficient sample). Among the 126 enrolled and investigated patients, there were 103 patients with laboratory confirmed DENV infection and 23 patients with other febrile illnesses that were not dengue (Table I). There were 92 (73%) patients who returned for follow-up 15–30 d after enrollment. After applying World Health Organization criteria (24) to each dengue case, there were 17 DHF patients and 86 DF patients.

Circulation of surface-activated CD8+ T cells and their relationship to viremia dynamics

There were 93 patients with a measurable viremia at enrollment, with DENV-1 the most prevalent serotype detected (49/93 [52%]) (Table I). Staining for intracellular markers of cellular proliferation (nuclear Ag Ki-67) and surface markers of activation (CD38+ and HLA-DR+) on CD3+CD8+ T cells were performed at enrollment and every second day on fresh whole blood samples. Results are shown for DENV-1 and DENV-3 only as the sample size for other serotypes was small. Double-positive CD3+CD8+ T cells (CD38+HLA-DR+, CD38+Ki-67+, or HLA-DR+Ki-67+) were increased in blood only when the DENV-1 (Fig. 1A, 1C, 1E) or DENV-3 (Fig. 1B, 1D, 1F) viremia had already begun to decline. Indeed, phenotypically activated CD8+ T cells were most prominent after defervescence, a time when most patients were afebrile. Collectively, these data suggest the commencement of clearance of viremia, and a sizable fraction of the resolved viremia, occurs in the absence of measurable peripheral blood T cell activation as defined by the activation markers used here.

CD8+ T cell activation and DENV-specific CD8+ T cell responses and their relationship to vascular leakage

Microvascular leakage is a signature feature of DHF. In DHF patients (∼17), hemococoncentration began during the febrile
phase and the median day at which it peaked was 1 d (FD = −1) before defervescence (Fig. 2A–C). In contrast, an increased (defined as twice the mean convalescent value) in the percentage of surface activated CD3+CD8+ T cells (CD38+HLA-DR+, CD38+Ki-67+, or HLA-DR+Ki-67+) was not measurable in DHF patients until a median FD of 1, (range, 2–4 to 3), and this was statistically significant for all three T cell populations (p < 0.001). This suggested hemoconcentration in DHF patients commenced in the absence of any increase in the percentage of surface activated CD8+ T cells in peripheral blood. We deliberately did not stratify patients further into their primary or secondary serological status.

Table I. Characteristics of the patient population investigated for CD8+ T cell phenotype and frequency of NS3133–142-specific T cells

<table>
<thead>
<tr>
<th>Variable</th>
<th>DF (n = 86) Median (Range) or No. (%)</th>
<th>DHF (n = 17) Median (Range) or No. (%)</th>
<th>Other Febrile Illness (n = 23) Median (Range) or No. (%)</th>
<th>p Value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex, no. (%)</td>
<td>40 (46.5)</td>
<td>12 (70.6)</td>
<td>14 (60.9)</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>11 (4–15)</td>
<td>13 (8–15)</td>
<td>11 (5–14)</td>
<td></td>
</tr>
<tr>
<td>Day of illness&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2 (1–4)</td>
<td>3 (1–4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FD&lt;sup&gt;c&lt;/sup&gt;</td>
<td>−3 (−5 to −1)</td>
<td>−3 (−6 to −1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infecting serotype, no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DENV-1</td>
<td>39 (51.3)</td>
<td>10 (58.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DENV-2</td>
<td>10 (13.2)</td>
<td>3 (17.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DENV-3</td>
<td>26 (34.2)</td>
<td>4 (5.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DENV-4</td>
<td>1 (1.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary infection, no. (%)</td>
<td>59 (68)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log&lt;sub&gt;10&lt;/sub&gt; of peak viremia, mean (range), cDNA copies/ml</td>
<td>7.37 (4.45–9.90)</td>
<td>7.81 (5.88–9.63)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Platelet nadir, cells/μl</td>
<td>116,000 (35,200–270,000)</td>
<td>47,100 (11,600–88,300)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Maximum hemoconcentration (%)</td>
<td>8 (−13 to 54)</td>
<td>27 (22–53)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Mann-Whitney U test.

<sup>b</sup>At time of study enrollment.

<sup>c</sup>The day of defervescence (axillary temperature) was regarded as FD 0.

FIGURE 1. Relationship between kinetics of viremia and the appearance of surface activated CD8+ T cells in peripheral blood. Shown in each panel are the mean (±95% CI) of DENV-1 (n = 49; A, C, E) or DENV-3 (n = 30; B, D, F) viremia levels in serial plasma samples from children by FD. The day of defervescence was defined as FD 0. In each panel, the box and whisker plots represent the percentage of CD8+ T cells that were double positive for CD38/HLA-DR (A, B), CD38/Ki-67 (C, D), or HLA-DR/Ki-67 (E, F). The number of patients that were evaluated on each day is shown below E and F.
for this analysis as the majority of DHF patients had secondary dengue (82%) and our primary end point of interest was capillary leakage.

To examine DENV-specific responses we used a pool of HLA-class I tetramers carrying common variants of the HLA-A*11-restricted NS3133–142 peptide from DENV-1, DENV-2, and DENV-3/4. The NS3133–142 epitope is typical of most CD8+ T cell epitopes in DENV in that across the four serotypes there are several variant sequences. Also typical is the presence of two or more variant sequences within a serotype. Supplemental Table I summarizes sequence diversity in known CD8+ T cell epitopes between and within serotypes in a global collection of 2148 DENV genome sequences sampled from 31 countries. To detect HLA-A*11-restricted NS3133–142 T cells, we stained whole blood samples (median, 4; range, 2–6 samples investigated per patient) from each study participant at enrollment and then every second day with a pool of tetramers. Of the 103 dengue patients, 33 patients (32%) had at least one sample with a measurable NS3133–142-specific tetramer staining population of CD8+ T cells, including nine dengue patients with DHF. The median FD on which tetramer positive cells were first detected in the nine DHF patients was +1 (range, −3 to 12) and this was 2 d later than the mean peak in hemoconcentration described for these patients in Fig. 2. Among the 33 patients who were ever tetramer positive, NS3133–142-specific T cells were detected during the febrile phase in less than half (13/33 [39%]) the cases. In the early convalescent afebrile phase, measurable NS3133–142-specific CD8+ T cells were present in a significantly greater proportion of patients who were ever positive (23/33 [70%]) (p = 0.02). The percentage of circulating NS3133–142-specific CD8+ T cells during the early convalescence phase was significantly higher than during the febrile phase (paired t test, p = 0.037) (Fig. 3). These results suggest NS3133–142-specific CD8+ T cells reach measurable frequencies around the time of defervescence and after the commencement of hemoconcentration in DHF patients.

Timing of DENV-specific CD8+ T cell responses in relationship to hematological markers of disease

To explore more fully the relationship between the appearance of measurable NS3133–142-specific T cells in blood and hemoconcentration and thrombocytopenia in a larger patient population, pooled tetramers were used to stain paired (study enrollment and early convalescent) peripheral blood samples from 422 children hospitalized with suspected dengue, of whom 390 were laboratory confirmed. The characteristics of this patient population are described in Table II. At enrollment, the median illness day was 4 d (range 1–6) and the median number of days prior to defervescence 2 d (range, −6 to 0). Among the 390 patients with laboratory confirmed dengue, there were 62 (16%) patients (41 with DHF and 21 with DF) with blood samples that were ever positive with the pooled NS3133–142-specific tetramers. All 62 patients were confirmed as being HLA-A*11-positive by PCR-specific sequence primers and 82% of the DHF patients had secondary dengue (Table II).

Daily hematocrit measurements indicated hemoconcentration (>20%) was first present a median of 1 d (FD range, −5 to 2 d)
prior to defervescence in the 41 patients with DHF that were ever positive with the pooled NS3133–142-specific tetramers (Fig. 4A).

Thrombocytopenia of <100,000 cells/mm³ was also first detected a median of 1 d (FD range: −5 to 2 d) prior to defervescence in the same patients (Fig. 4B). It is probable that hemoconcentration (>20%) and thrombocytopenia (<100,000 cells/mm³) occurred prior to study enrollment in some patients, leading to an overestimate of the time required for these hematological changes to first occur. In contrast, NS3133–142-specific CD8⁺ T cells first became detectable in DHF patients on FD +1 (FD range, −3 to 4 d) (Fig. 5A), a median of 2 d (FD range, 0 to 5 d) after thrombocytopenia (<100,000 cells/mm³) was first detected (comparison of median fever day, \( p = 0.0001 \) (Fig. 5B) and a median of 2 d (FD range, 0 to 5 d) after hemoconcentration (>20%) was first detected in each patient (comparison of median FD, \( p = 0.0001 \)) (Fig. 5C). The timing of these events is summarized in Table III. Collectively, these data indicate NS3133–142⁺ specific CD8⁺ T cells generally become measurable in the peripheral blood only after the commencement of vascular leakage or thrombocytopenia in patients with DHF.

# Discussion

Secondary heterotypic DENV infections are a risk factor for DHF in children and adults. Anemestic, cross-reactive T cells responses are a part of the host response to secondary infection and have been suggested to contribute to immunopathogenesis. The major findings in the current study are that surface activated CD8⁺ T cells and NS3133–142⁺ specific CD8⁺ T cells are generally not measurable in peripheral blood prior to the commencement of hemoconcentration, thrombocytopenia, or resolution of viremia and therefore it seems unlikely they are critical triggers of these events.

Significant immune activation undoubtedly occurs in patients with DHF and secondary infections. Relatively higher levels of various proinflammatory cytokines and their receptors and soluble CD4/8 are found in acute sera of children with DHF compared with children with DF (18, 30, 31). DHF patients also have a greater percentage of CD8⁺ T cells bearing the early activation marker CD69⁺ than DF patients during the febrile phase (19). In the context of virus-specific responses, Mongkolsapaya et al. detected very low frequencies of NS3133–142⁺ specific T cells in the late febrile phase with a subsequent peak in frequency a few weeks after illness onset (21). The very low-frequency of NS3133–142⁺ specific CD8⁺ T cells in the late febrile phase was suggested to reflect huge proliferation balanced by massive apoptosis (21). However, these findings were not analyzed in relation to the commencement of capillary leakage. Our own previous studies have indicated DENV-epitope–specific T cells are difficult to detect during the febrile phase by ELISPOT assay, but that responses are readily measured in early convalescence (20). Other studies of virus-specific responses demonstrated ELISPOT frequencies of T cells specific for the HLA-B*7–restricted epitope NS322–230 were

### Table II. Characteristic of the patient population in whom tetramer staining was performed for NS3133–142-specific T cells

<table>
<thead>
<tr>
<th>Variable</th>
<th>DF (n = 21) Median (Range)/No. (%)</th>
<th>DHF (n = 41) Median (Range)/No. (%)</th>
<th>Dengue (n = 328) Median (Range)/No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex, no. (%)</td>
<td>13 (61.9)</td>
<td>25 (60.9)</td>
<td>176</td>
</tr>
<tr>
<td>Age (y)</td>
<td>10 (3–14)</td>
<td>10 (2–15)</td>
<td>10 (1–15)</td>
</tr>
<tr>
<td>Day of illness at enrollment</td>
<td>3 (1–4)</td>
<td>3 (1–6)</td>
<td>4 (2–7)</td>
</tr>
<tr>
<td>FD at enrollment⁹</td>
<td>−2 (−3 to 0)</td>
<td>−2 (−5 to 0)</td>
<td>−2 (−6 to 0)</td>
</tr>
<tr>
<td>Infecting serotype, no. (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DENV-1</td>
<td>4 (22.2)</td>
<td>12 (31.6)</td>
<td>186 (63.9)</td>
</tr>
<tr>
<td>DENV-2</td>
<td>10 (55.6)</td>
<td>19 (50)</td>
<td>83 (28.5)</td>
</tr>
<tr>
<td>DENV-3</td>
<td>4 (22.2)</td>
<td>7 (18.4)</td>
<td>21 (7.2)</td>
</tr>
<tr>
<td>DENV-4</td>
<td>0</td>
<td>0</td>
<td>1 (0.3)</td>
</tr>
<tr>
<td>Secondary infection, no. (%)</td>
<td>18 (85.7)</td>
<td>34 (82.9)</td>
<td>247</td>
</tr>
<tr>
<td>Log₁₀ of viremia, mean (range), cDNA copies/ml⁹</td>
<td>6.82 (3.98–9.77)</td>
<td>7.49 (3.78–12.21)</td>
<td>6.80 (3.03–11.83)</td>
</tr>
<tr>
<td>Platelet nadir, cells/μl</td>
<td>61,000 (33,000–124,000)</td>
<td>36,000 (10,000–96,000)</td>
<td>50,000 (1,000–270,000)</td>
</tr>
<tr>
<td>Maximum hemoconcentration (%)</td>
<td>13.9 (2.2–19.4)</td>
<td>28 (10.5–54.1)</td>
<td>22 (22.6 to 52.78)</td>
</tr>
</tbody>
</table>

Data are median (range) values, unless otherwise indicated.

⁹The day of defervescence was regarded as fever day 0.

⁹DENV viremia measured at enrollment.
In two populations of children with DHF we found that NS3133–142-specific CD8+ T cell responses and hemoconcentration, thrombocytopenia and defervescence in children with dengue. Shown in each scatterplot is the percentage of NS3133–142-specific CD8+ T cell detected by tetramer staining in individual patient blood samples against a reference timepoint of day of defervescence (day 0 in A), day when platelet count of <100,000 cells/μl was first detected in each patient (day 0 in B), and day when hemoconcentration of >20% was first detected in each patient (day 0 in C). The values below the x-axis are the number of patients evaluated on each day.

There are several limitations to our study. First, our investigations of surface markers on CD8+ T cells was not exhaustive but focused on two well-described markers of activation (HLA-DR and CD38) and one intracellular marker of cellular proliferation (Ki-67). It is possible that T cells responding to altered peptide ligands in vivo may not express these surface markers or enter cell cycle and this should be a focus for future research. Secondly, we investigated CD8+ T cells only when T regulatory and conventional CD4+ T cells could also be important (35–37). Third, our analysis of DENV-specific CD8+ T cells was limited to early convalescence and this was consistent with previous studies that have used this marker (21). NS3133–142-specific CD8+ T cells were also rarely detected during the febrile phase, and significantly, were generally not measurable prior to the detection of hemoconcentration (>20%) or thrombocytopenia in children with DHF. The temporal mismatch between the first detection of hemoconcentration and measurable CD8+ T cells or NS3133–142-specific CD8+ T cells might suggest these cells have a negligible role in triggering capillary leakage. An alternative explanation for the absence or very low frequency of NS3133–142-specific T cells during the febrile phase [this study and (21)], is that they are sequestered in sites of infection or have downregulated TCRs (29). This would imply a massive expansion and distribution of central and/or effector memory NS3133–142-specific CD8+ T cells after a few days of infection and negligible ongoing circulation of CD8+ T cells expressing the relevant, specific TCR. A second alternative explanation is that they are sequestered and proliferating in lymphoid tissue during the febrile phase but have yet to enter the peripheral circulation. Studies to address these issues, in either primate animal models or more directly in patients (e.g., fine biopsy collections), will be needed to resolve these questions. A plausible role for T cells in the immunopathogenesis of secondary dengue is that they act to amplify an already established proinflammatory cascade mediated by innate responses to a large viral burden. In this two-stage model, a large viral Ag mass (plausibly mediated by Ab-dependent enhancement) might be necessary and sufficient to trigger capillary leakage through innate mechanisms, such as complement activation, cytokine secretion by activated macrophages/dendritic cells, and/or NS1-mediated perturbations of the vascular endothelium. Exacerbation of capillary leakage that has already commenced might occur when effector T cells accumulate at sites of infection. Such a model could also explain DHF in infants with primary infections and no DENV-specific memory T cells; the innate response (complement activation, cytokine secretion) may be necessary and sufficient to trigger clinically significant capillary leakage in infants because of their intrinsically poor capacity to compensate for microvascular leakage compared with older children or adults (32). Consistent with this, many of the cytokines found to be elevated in children with DHF and secondary infection are also elevated in infants with primary infection (33, 34).

There are several limitations to our study. First, our investigations of surface markers on CD8+ T cells was not exhaustive but focused on two well-described markers of activation (HLA-DR and CD38) and one intracellular marker of cellular proliferation (Ki-67). It is possible that T cells responding to altered peptide ligands in vivo may not express these surface markers or enter cell cycle and this should be a focus for future research. Secondly, we investigated CD8+ T cells only when T regulatory and conventional CD4+ T cells could also be important (35–37). Third, our analysis of DENV-specific CD8+ T cells was limited to early convalescence and this was consistent with previous studies that have used this marker (21). NS3133–142-specific CD8+ T cells were also rarely detected during the febrile phase, and significantly, were generally not measurable prior to the detection of hemoconcentration (>20%) or thrombocytopenia in children with DHF. The temporal mismatch between the first detection of hemoconcentration and measurable CD8+ T cells or NS3133–142-specific CD8+ T cells might suggest these cells have a negligible role in triggering capillary leakage. An alternative explanation for the absence or very low frequency of NS3133–142-specific T cells during the febrile phase [this study and (21)], is that they are sequestered in sites of infection or have downregulated TCRs (29). This would imply a massive expansion and distribution of central and/or effector memory NS3133–142-specific CD8+ T cells after a few days of infection and negligible ongoing circulation of CD8+ T cells expressing the relevant, specific TCR. A second alternative explanation is that they are sequestered and proliferating in lymphoid tissue during the febrile phase but have yet to enter the peripheral circulation. Studies to address these issues, in either primate animal models or more directly in patients (e.g., fine biopsy collections), will be needed to resolve these questions. A plausible role for T cells in the immunopathogenesis of secondary dengue is that they act to amplify an already established proinflammatory cascade mediated by innate responses to a large viral burden. In this two-stage model, a large viral Ag mass (plausibly mediated by Ab-dependent enhancement) might be necessary and sufficient to trigger capillary leakage through innate mechanisms, such as complement activation, cytokine secretion by activated macrophages/dendritic cells, and/or NS1-mediated perturbations of the vascular endothelium. Exacerbation of capillary leakage that has already commenced might occur when effector T cells accumulate at sites of infection. Such a model could also explain DHF in infants with primary infections and no DENV-specific memory T cells; the innate response (complement activation, cytokine secretion) may be necessary and sufficient to trigger clinically significant capillary leakage in infants because of their intrinsically poor capacity to compensate for microvascular leakage compared with older children or adults (32). Consistent with this, many of the cytokines found to be elevated in children with DHF and secondary infection are also elevated in infants with primary infection (33, 34).
one dominant epitope (NS3133-142) restricted through the most common class I HLA allele in the Vietnamese population, HLA-A*11. Reponses to other DENV CDS3 T epitopes might occur with greater rapidity than that described in this study. Finally, our study was biased toward patients with DENV-1 as this was the most prevalent serotype in circulation at the time. Nevertheless, this study provides the first description of a temporal mismatch between the CDS3 T cell response to a well-characterized epitope and commencement of capillary leakage. Future studies of T cell responses in dengue need to consider the timing of responses relative to the commencement of capillary leakage, generally the most important clinical complication of dengue.

Disclosures

The authors have no financial conflicts of interest.

References


