Dengue-Specific T Cell Responses in Peripheral Blood Mononuclear Cells Obtained prior to Secondary Dengue Virus Infections in Thai Schoolchildren

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Children who experience secondary dengue virus (DV) infections are at increased risk for dengue hemorrhagic fever. To study the effect of preexisting T cell responses to DV on the severity of secondary virus infection, peripheral blood mononuclear cells (PBMC) from 10 subsequently hospitalized and 12 nonhospitalized Thai schoolchildren were stimulated with inactivated dengue antigens, and proliferation of interferon (IFN)–γ or tumor necrosis factor (TNF)–α responses of the preinfection PBMC were measured. Proliferation responses were observed in 11 subjects, and IFN-γ responses were seen in 12 subjects, 6 of whom showed broad serotype cross-reactive IFN-γ responses. TNF-α responses were detected exclusively in 4 hospitalized subjects. Four PBMC samples that showed neither proliferation nor cytokine responses to any dengue antigen were from nonhospitalized subjects. This study, thought to be the first to investigate T cell responses to DV in preinfection PBMC, suggests that the pattern of preexisting T cell responses influences the risk for severe disease.

Dengue fever (DF) and the more severe dengue hemorrhagic fever (DHF) are important global public health problems among the new and newly emerging infectious diseases [1]. The dengue viruses (DVs), the causative agents of DF and DHF, are considered to be the most important arthropodborne viruses in terms of morbidity and mortality in tropical and subtropical regions [2–5]. There are 4 closely related but serologically distinct DVs, dengue serotypes 1–4. Infection with 1 may confer lifelong immunity to that serotype but not to heterologous serotypes [4].

DHF is manifested by fever with plasma leakage and thrombocytopenia, often accompanied by hepatomegaly and, in severe cases, circulatory failure. An estimated half million cases of DHF are reported each year, and mortality rates in untreated cases are high [5, 6]. Epidemiologic studies show an increased risk for the occurrence of DHF in subjects who experience secondary DV infections [7–9]. DVs are consistently associated with mononuclear phagocyte cells in autopsy samples. In vitro, DV infection of monocytes is enhanced by DV-specific antibodies [10, 11]. These observations suggest the involvement of host immune factors and an immunopathogenetic mechanism in the development of DHF [12–15]. Our laboratory has demonstrated the existence of dengue serotype-specific and cross-reactive CD4 and CD8 T cells [16–28]. Other studies have also shown significantly higher serum or plasma levels of soluble (s) receptors, such as sCD8, soluble interleukin-2 receptor, and soluble tumor necrosis factor (TNF) receptor II, in children with DHF than in those with DF [22, 29, 30]. Increased expression of the activation marker CD69 on CD8 T cells was demonstrated during acute DV infection and was significantly higher in DHF than in DF during the early stages of the infection [31].

Our model of the immunopathogenesis of plasma leakage in DHF predicts the involvement of vasoactive cytokines secreted by activated dengue-specific T lymphocytes and infected monocytes in capillary permeability [2]. We further hypothesize that the degree of activation of dengue-specific memory T cells derived from a primary dengue infection affects the severity of symp-
Materials and Methods

Study design and sample collection. The design of this prospective study will be reported elsewhere. In brief, ~2400 primary school students in Kamphaeng Phet Province, Thailand, were enrolled in the first year of this ongoing study. A blood sample was collected from all subjects in January 1998, and plasma and PBMC were separated and cryopreserved. During the peak period of dengue transmission (June–November), the subjects were under daily active surveillance for school absences associated with fever. Absentees who met specific criteria (fever or history of fever within 7 days of the first day of school absence) were evaluated, and acute and convalescent (15-day) blood samples were collected.

DV infections were identified by virus isolation and/or reverse-transcriptase polymerase chain reaction from acute serum. Secondary dengue infections were determined by dengue IgG/IgM ELISA and hemagglutination inhibition (HI) tests against all 4 dengue serotypes [32, 33]. All laboratory-confirmed virus infections were characterized according to World Health Organization (WHO) guidelines for DF and DHF [34]. Blood samples were collected from all subjects in June, August, and November and were tested by HI to identify asymptomatic DV infections (>4-fold increase in HI titer from June–August or August–November).

Characteristics of study population. We studied 22 schoolchildren from 1 primary school that experienced a dengue 3 outbreak. This information is summarized in table 1. Ten subjects were hospitalized during the acute DV infection, and 9 were not hospitalized but experienced overt symptoms of dengue. Three of the hospitalized subjects (nos. 392, 534, and 712) were admitted to the hospital with a presumptive diagnosis of DHF but, upon subsequent inde-

Table 1. Presecondary infection characteristics of study population.

<table>
<thead>
<tr>
<th>Subject group, no.</th>
<th>Age/sex/JE vaccination status</th>
<th>Plaque reduction neutralization titers</th>
<th>Den IgM/IgG&lt;sup&gt;a&lt;/sup&gt;</th>
<th>JE IgM/IgG&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonhospitalized</td>
<td>Den 1</td>
<td>Den 2</td>
<td>Den 3</td>
<td>Den 4</td>
</tr>
<tr>
<td>479</td>
<td>N&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7/M/N</td>
<td>910</td>
<td>&lt;10</td>
</tr>
<tr>
<td>565</td>
<td>N&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8/F/Y</td>
<td>35</td>
<td>&lt;10</td>
</tr>
<tr>
<td>701</td>
<td>N&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10/F/Y</td>
<td>2815</td>
<td>178</td>
</tr>
<tr>
<td>453</td>
<td>N</td>
<td>7/F/U</td>
<td>1539</td>
<td>2964</td>
</tr>
<tr>
<td>481</td>
<td>N</td>
<td>7/M/N</td>
<td>253</td>
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</tr>
<tr>
<td>490</td>
<td>N</td>
<td>7/F/N</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>506</td>
<td>N</td>
<td>8/M/Y</td>
<td>25</td>
<td>11</td>
</tr>
<tr>
<td>520</td>
<td>N</td>
<td>8/F/Y</td>
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<td>64</td>
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<tr>
<td>617</td>
<td>N</td>
<td>8/F/N</td>
<td>9914</td>
<td>536</td>
</tr>
<tr>
<td>636</td>
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<td>704</td>
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<tr>
<td>724</td>
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<td>&lt;10</td>
</tr>
<tr>
<td>Hospitalized</td>
<td>Den 1</td>
<td>Den 2</td>
<td>Den 3</td>
<td>Den 4</td>
</tr>
<tr>
<td>392</td>
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<td>7/M/U</td>
<td>&lt;10</td>
<td>&lt;10</td>
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<tr>
<td>534</td>
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<tr>
<td>712</td>
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<td>409</td>
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<td>49</td>
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<td>7/M/U</td>
<td>1137</td>
<td>&lt;10</td>
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<tr>
<td>517</td>
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<td>8/M/Y</td>
<td>61</td>
<td>122</td>
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<tr>
<td>648</td>
<td>Y</td>
<td>9/F/Y</td>
<td>&lt;10</td>
<td>95</td>
</tr>
<tr>
<td>675</td>
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<td>9/F/Y</td>
<td>240</td>
<td>36</td>
</tr>
<tr>
<td>715</td>
<td>Y</td>
<td>10/F/N</td>
<td>&lt;10</td>
<td>299</td>
</tr>
</tbody>
</table>

NOTE. Den, dengue; DHF, dengue hemorrhagic fever; JE, Japanese encephalitis; N, no; ND, not determined; U, unknown; Y; yes.

<sup>a</sup>Met World Health Organization criteria for DHF.

<sup>b</sup>IgM/IgG values are after secondary infection. IgM/IgG < 1.78 is considered to be a secondary infection [33].

<sup>c</sup>Subjects experienced a 4-fold increase in dengue antibody titer without associated febrile school absence.
pended thymidine ([3H]TdR) for 12 h. Cells were harvested by multi-
dergent or control antigens at 3 dilutions (1:40, 1:80, and 1:160) in
hospitalized persons was done by Fisher’s exact test. Compar-
serotypes, between June and August 1998 and an increase in neutralizing antibody titers to dengue 3 from January and November of 1998. Four subjects (nos. 490, 724, 392, and 534) did not have any detectable preinfection neutralizing antibody titers to dengue, but the dengue IgM/IgG response to acute DV infection was indicative of a secondary den-
gue infection.

Preparation of viral antigens. Viral antigens for each of the 4
dengue serotypes were prepared as lysates of DV-infected Vero
cells, as described elsewhere [23]. Control antigens were prepared in a similar fashion by using uninfected Vero cells.

Lymphocyte proliferation assays. We cultured 10^5 PBMC with
dengue or control antigens at 3 dilutions (1:40, 1:80, and 1:160) in
200 µL of RPMI 1640 medium containing 10% heat-inactivated human AB serum in 96-well round-bottom plates at 37°C for 6
days. Cultures were done in triplicate for each specific antigen
dilution. Because of limited numbers of PBMC, some samples were tested at only 1 (1:40) or 2 (1:40 and 1:80) dilutions of anti-
gen. We collected 100 µL of culture supernatant from each well 12 h before harvest; triplicates were pooled and frozen for subsequent
cytokine assays. The cells were then pulsed with 1.25 µCi of tri-
tiated thymidine ([3H]TdR) for 12 h. Cells were harvested by multi-
harvester (Titertek; Skatron), and [3H]TdR incorporation was measured in a liquid scintillation counter (Packard Instrument).
Dengue-specific proliferation was expressed as follows: stimulation index (SI) = incorporated [3H]TdR in dengue antigen–stimu-
lated PBMC/incorporated [3H]TdR in control antigen–stimulated
PBMC. SI > 3 was defined as a positive proliferation response.

Measurement of antigen-induced cytokines. PBMC were stimu-
lated with dengue antigens or control antigen, and culture super-
natants were collected, as described above. Cytokines secreted by
cultured PBMC were measured by commercial ELISA kits for inter-
erferon (IFN)–γ and TNF-α (Endogen). The assays were per-
formed according to the manufacturer’s instructions. The limit of de-
tection for each assay was 2 pg/mL. A cytokine concentration > 10 pg/mL above background (control antigen–stimulated PBMC) was
considered to be a positive response. This value is > 3 times the SD
from the mean of background as well as negative control supernatant
cytokine values. Data shown are values above background.

Statistical analysis of proliferation and cytokine data. Com-
parison of mean SIs and IFN-γ and TNF-α concentrations between
hospitalized and nonhospitalized subjects was done by Student’s t
test. Nonhospitalized symptomatic and asymptomatic groups were
combined, although results were similar when the nonhospitalized
symptomatic group alone was considered. Comparison of the pro-
portion of responders in each assay between hospitalized and non-
hospitalized persons was done by Fisher’s exact test.

Results

Lymphoproliferation responses to antigen stimulation. Fig-
ure 1 shows the proliferation responses of PBMC against 3 di-
lutions of the 4 different dengue antigens. PBMC from 11 subjects
showed a proliferation response to ≥ 1 of the 4 dengue serotype
antigens (table 2). Positive SI values were 3.1–35. Mean SI values
were highest against dengue 1. Serotype cross-reactive responses
were seen in 9 subjects: 1 subject responded to all 4 antigens; 3
subjects responded to dengue 1, 2, and 3; 3 subjects responded to
dengue 1 and 3; 1 subject responded to dengue 2 and 3; and 1 sub-
ject responded to dengue 1 and 2. Of the 2 subjects with serotype-
specific responses, 1 responded only to dengue 1, and 1 responded
only to dengue 2. Seven subjects had proliferation against den-
gue 3 antigen.

IFN-γ secretion of PBMC after antigen stimulation. Figure 2
shows the IFN-γ responses of PBMC against 3 dilutions of the 4
different dengue antigens. IFN-γ secretion was detected in 12
subjects (table 2). Positive dengue-specific IFN-γ values were
10–2498 pg/mL. Serotype cross-reactive responses were seen in 10
subjects, of whom 6 responded to all serotypes. Two sub-
jects showed IFN-γ responses to dengue 1 and 3 antigens. One
subject responded to dengue 2 and 3 antigens, and another re-
responded to dengue 3 and 4 antigens. Two subjects had a dengue
1–specific IFN-γ response.

Figure 1. Lymphocyte proliferation responses to 4 dengue (den) se-
rotypes of presecondary infection peripheral blood mononuclear cells
(PBMC) from 22 Thai schoolchildren. Ten were hospitalized during
the subsequent secondary infection, and 12 did not require hospitali-
zation (9 symptomatic and 3 asymptomatic). PBMC were incubated
with indicated dengue antigens and antigen dilutions for 6 days. Cells
were pulsed with tritiated thymidine for 12 h before harvest. Stimu-
lation index (SI) was calculated as mean counts per minute (cpm) in-
duced by dengue antigen/mean cpm induced by control antigen.
SI > 3 was considered to be positive (dashed line).
The percentage of IFN-γ nonhospitalized subjects (4 [40%] of 10 vs. 7 [58.3%] of 12), whereas any serotype was lower in the hospitalized subjects than in the nonhospitalized subjects. Differences between the hospitalized and nonhospitalized groups. Similarly, mean values of presecondary infection PBMC between hospitalized and nonhospitalized subjects. Mean proliferation values against antigens, respectively, and another showed responses to dengue antigens, respectively, and another showed responses to dengue 1 and 2. Dengue 1 and 4–specific responses were seen in the hospitalized and nonhospitalized groups. The percentage of IFN-γ responders who reacted to all 4 serotypes in the hospitalized group than in the nonhospitalized group (2 [28.6%] of 7 vs. 4 [80%] of 5). These differences were not statistically significant.

All 4 subjects with TNF-α responses were in the hospitalized group (P = .029). Four subjects (nos. 565, 481, 506, and 636) without detectable proliferation, IFN-γ, or TNF-α responses were in the nonhospitalized group (P = .096).

Although some subjects showed a correlation between high proliferation and cytokine responses, this did not hold true for all study subjects: 5 (2 hospitalized and 3 nonhospitalized) showed proliferation but no cytokine responses; 6 subjects (5 hospitalized and 1 nonhospitalized) showed cytokine but no proliferation responses.

**Discussion**

Earlier studies evaluated T cell responses to DV in PBMC samples taken after secondary infections or in subjects who did not have a subsequent secondary dengue infection to allow correlation with the disease outcome. The prospective design of the present study allowed us to investigate cellular immune responses to primary dengue infections and to correlate these with the disease outcome of the secondary dengue infection.

We measured preinfection T cell proliferation and cytokine responses after stimulation with antigens from all 4 dengue serotypes and correlated these with the severity of the subsequent secondary dengue infection by using the physician’s decision to hospitalize the patient as the main indicator of severity. Although 3 hospitalized subjects did not meet WHO criteria for

**Table 2.** Summary of T cell responses to dengue virus in peripheral blood mononuclear cells obtained prior to secondary dengue virus infections in the study cohort.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All (n = 22)</th>
<th>Hospitalized (n = 10)</th>
<th>Nonhospitalized symptomatic (n = 9)</th>
<th>Nonhospitalized asymptomatic (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any responsea</td>
<td>18</td>
<td>10</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Proliferationb</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>For any serotype</td>
<td>11</td>
<td>4</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>&gt; 1 Serotype</td>
<td>9</td>
<td>3</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Dengue 3</td>
<td>7</td>
<td>2</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>IFN-γ secretionc</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>For any serotype</td>
<td>12</td>
<td>7</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>&gt; 1 Serotype</td>
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<td>2</td>
</tr>
<tr>
<td>Dengue 3</td>
<td>9</td>
<td>5</td>
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<td>2</td>
</tr>
<tr>
<td>TNF-α secretionc</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>For any serotype</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>&gt; 1 Serotype</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dengue 3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

NOTE. IFN, interferon; TNF, tumor necrosis factor.

a Proliferation and/or cytokine response.
b Stimulation index > 1.
c Cytokine level > 10 pg/mL above negative control.

**Fig. 2.** Levels of interferon (IFN)-γ in antigen-stimulated peripheral blood mononuclear cell (PBMC) culture supernatants. Culture supernatants were collected 6 days from antigen-stimulated PBMC (see figure 1 legend). IFN-γ was measured by using ELISA kits. IFN-γ > 10 pg/mL above background control levels were considered to be positive (dashed line). Values shown are above background.
Dengue, these subjects were not studied according to a fixed protocol. Early hospital admission and fluid therapy may therefore have limited hemoconcentration leading to an under diagnosis of DHF.

In the present study, we found no direct correlation between dengue 3–specific proliferation, IFN-γ or TNF-α responses, and severity of the secondary dengue infection. The absence of TNF-α responses to dengue 3 antigen (figure 3 and table 2) was unexpected, given that dengue 3 was the serotype of the secondary infection experienced by all of the symptomatic subjects. However, the dengue 3 antigen used was derived from dengue 3 strain CH53489, a 1973 isolate from Bangkok. Genetic variation between this strain and the Thai dengue 3 strain circulating in the study area in 1998 might have affected the in vitro responses, although we have not seen differences in dengue-specific T cell proliferation (unpublished data) or cytokine secretion [35] in other dengue-immune persons related to the virus used for preparation of antigens.

The varied patterns of T cell responses in these subjects indicate that the host response to a primary dengue infection is complex and that different patterns of proliferation and cytokine production may bring about the same disease outcome. Interactions between the preexisting T cell response and other host and viral factors may be important in determining the disease outcome. Serotype cross-reactive nonneutralizing antibodies can lead to increased uptake of DV by Fc receptor–bearing cells [12] with resulting increased antigen presentation and induction of inflammatory cytokines that cause vascular leakage [3]. Plasma virus titers correlate with disease severity in secondary dengue 2 [36] and dengue 3 [37] infections. In the latter study, plasma viral RNA levels were more strongly associated with disease severity than markers of immune activation.

Although we found no significant differences between groups in mean proliferation or cytokine responses, several preliminary differences were noted. While serotype cross-reactive IFN-γ responses were seen in both hospitalized and nonhospitalized groups, there was a tendency for IFN-γ responses to be more broadly serotype cross-reactive in nonhospitalized subjects (table 3). Although IFN-γ can augment antibody-dependent enhancement [38], it also has antiviral activity and can enhance activation and IFN-γ and TNF-α production by DV-infected dendritic cells [39]. These activities could contribute to earlier virus clearance and reduced disease severity.

A second observation was that all of the subjects with TNF-α responses to dengue antigen were in the hospitalized group. Earlier studies from our laboratory and from other investigators detected elevated plasma levels of soluble TNF-α receptors more often during acute infection in patients with DHF than in those with DF [29, 40, 41]. We previously showed that some dengue-specific CD4 T cells produce TNF-α in response to stimulation in vitro [35, 42]. Our results here suggest that dengue-specific TNF-α production by memory T cells is a risk factor for the occurrence of severe dengue disease in Thai schoolchildren. These observations might be useful clinically in identifying persons who may be predisposed to DHF. However, further studies are necessary to confirm this association.

Of note, the 4 subjects who showed neither proliferation nor cytokine responses to any of the dengue antigens were from the nonhospitalized group. All 4 had detectable preinfection neutralizing antibody titers to DV, confirming prior DV infection. These observations might be useful in identifying persons

![Table 3. Interferon-γ responses to dengue virus in hospitalized and nonhospitalized subjects (in pg/mL).](https://academic.oup.com/jid/article-abstract/185/12/1697/900338)

<table>
<thead>
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<th>Patient group, no.</th>
<th>Dengue antigen</th>
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</tr>
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<td>2498</td>
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<td>27</td>
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<tr>
<td>715</td>
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<tr>
<td><strong>Nonhospitalized</strong></td>
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</tr>
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<td>87</td>
</tr>
<tr>
<td>704</td>
<td>147</td>
</tr>
</tbody>
</table>

*Not shown: 3 hospitalized and 7 nonhospitalized subjects who had negative interferon-γ responses.*

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**Figure 3.** Levels of tumor necrosis factor (TNF)-α in antigen-stimulated peripheral blood mononuclear cell (PBMC) culture supernatants. Culture supernatants were collected after 6 days from antigen-stimulated PBMC (see figure 1 legend). TNF-α was measured by using ELISA kits. TNF-α >10 pg/mL above background control levels were considered to be positive (dashed line). Values shown are above background.
who may be less susceptible to DHF. The lack of T cell responses after primary infection in these subjects may be explained as either the CD4 T cell responses elicited by the primary dengue infection in this group of subjects may have been very low and/or the CD4 T cell responses may have waned over time. This suggests that a diminished level of memory CD4 T cell Th1 activity may spare the host from a severe secondary infection.

Several limitations were encountered in the course of this study. First, the small number of preinfection PBMC we could obtain from children for the in vitro studies confined our measurements to a small number of antigens and dilutions. We were therefore unable to test the responses from other flaviviruses, such as the JE virus, which is known to circulate in Thailand. In addition, we were not able to measure the production of other cytokines that might have been secreted during antigen stimulation. Second, we used inactivated viral antigens to stimulate T cells in vitro. As a result, our measurements of memory T cells in the present study focused on CD4 T cells [23]. Further studies are needed to address the association of disease severity with CD8 T cell responses by using live DVs or viral vectors expressing dengue antigens. In addition, the use of intracellular cytokine staining and flow cytometry as well HLA peptide tetramers would allow the direct measurement of the preinfection frequency of dengue-specific T cells.

We believe this study is the first to analyze the relationship between cellular immune responses from a primary dengue infection and the severity of the secondary infection in the same subject. The correlation between TNF-α responses or the absence of both proliferation and cytokine responses and the subsequent hospitalization status of these subjects may be important in predicting which persons are or are not at risk for DHF.

Acknowledgments

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References