Intracellular Cytokine Production by Dengue Virus–specific T cells Correlates with Subclinical Secondary Infection

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The pathophysiology of dengue virus infection remains poorly understood, although secondary infection is strongly associated with more severe disease. In the present study, we performed a nested, case-control study comparing the responses of pre-illness peripheral blood mononuclear cells between children who would subsequently develop either subclinical or symptomatic secondary infection 6–11 months after the baseline blood samples were obtained and frozen. We analyzed intracellular cytokine production by CD4$^+$ and CD8$^+$ cells in response to stimulation with dengue antigen. We found higher frequencies of dengue virus–specific TNFα, IFNγ-, and IL-2–producing T cells among schoolchildren who subsequently developed subclinical infection, compared with those who developed symptomatic secondary dengue virus infection. Although other studies have correlated immune responses during secondary infection with severity of disease, to our knowledge this is the first study to demonstrate a pre-infection dengue-specific immune response that correlates specifically with a subclinical secondary infection.

Dengue is an important global health problem, and the dengue viruses (DENV) are the most prevalent arthropod-borne viruses in the tropics and subtropics today. There are an estimated 50–100 million DENV infections annually worldwide [1]. The spectrum of disease ranges from subclinical infection to classical dengue fever to the more severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). DHF and DSS, characterized by significant plasma leakage and occasionally hemorrhage and shock, account for an estimated half million cases and >20,000 deaths annually [2].

There are 4 distinct serotypes of dengue virus (DENV 1–4); infection with 1 serotype is believed to confer lifelong immunity to that serotype, whereas protective immunity to heterologous serotypes is short-lived, probably on the order of months [3]. One of the unique features of dengue is the role of heterologous infection in disease severity. Both clinical and epidemiological studies have suggested that the immune response plays a major role in the pathogenesis of severe dengue disease. Subsequent infection with a heterologous serotype predisposes patients toward more severe disease; studies in Southeast Asia, done both in the 1980s [4, 5] and more recently [6, 7], have shown that DHF is associated with secondary infection, implying that other mechanisms in addition to viral replication could be the cause of DHF.

Pre-existing heterotypic immunity is believed to increase disease severity in secondary DENV infection and, thus, the risk for DHF through 2 broad hypothetical (and not necessarily mutually exclusive) pathways.

The Antibody Dependent Enhancement hypothesis proposes that pre-existing heterotypic anti-DENV IgG antibodies acquired during a previous infection can, under certain conditions, facilitate uptake of virus into macrophages and other immune cells through the binding of virus-antibody complexes to Fcγ receptors. The consequent increase in receptor-mediated endocytosis leads to higher viral loads, which in turn, triggers a host inflammatory cascade that leads to DHF [8]. The second hypothesis involves cross-reactive memory T cells, which are activated during heterologous secondary infection. This leads to altered effector responses, including the generation of cytokines that may create an immunologic imbalance in response to infection and, thus, play either a protective or harmful role in the development of sequelae of infection [9].

Our model of T cell–mediated immunopathogenesis of DENV infection postulates that the relative production of vasoactive cytokines by activated dengue-specific memory T lymphocytes influences the severity of dengue illness [10]. To our knowledge, no previous studies have looked at host-immune factors that specifically correlate with subclinical dengue infection. We hypothesized that the memory T cell response generated during primary DENV infection is a major determinant of cytokine production during secondary infection and, therefore, influences the severity of symptoms experienced during secondary infection. Specifically, we predicted that the frequencies of dengue-specific, cytokine-producing T cells in peripheral blood mononuclear cells (PBMCs) obtained before secondary infection would correlate with the severity of disease seen in secondary infection.

To address this hypothesis, we tested PBMCs from Thai schoolchildren enrolled in a prospective cohort study [11]. The unique aspects of this study included the prospective scheduled collection and cryopreservation of PBMCs and the active surveillance for all febrile illnesses. This cohort provided an opportunity to study the predictive value of pre-infection cellular immune profiles with disease phenotype after subsequent DENV infection. From PBMCs obtained before secondary infection, we measured dengue-specific CD4$^+$ and CD8$^+$ T cell cytokine responses to dengue antigens to assess whether these responses predicted future disease severity. Because of the short interval between the collection of the baseline and follow-up blood samples, we believe that the frequencies of the DENV-specific cells at baseline reflect, as accurately as is possible in a large-scale prospective cohort, the host immune status with respect to DENV at the time of secondary infection.

MATERIALS AND METHODS

Study Design
We performed a nested, case-control study of 33 schoolchildren who were known to have developed secondary infection as part of a prospective study of primary school students in Kamphaeng

Phet Province, Thailand. Using PBMCs obtained at the beginning of the enrollment period when all participants were healthy, we compared the frequency of intracellular cytokine-producing cells after stimulation with dengue antigens between 2 groups of children: one that had symptomatic dengue infection and a control group that had subclinical infection. The design of the prospective study has been reported elsewhere [11]. In brief, blood samples were collected from all participants in January 1998; plasma samples and PBMCs were cryopreserved and stored in liquid nitrogen. In addition, blood samples were collected from all participants in June, August, and November and were tested by hemagglutination inhibition (HI) to identify subclinical DENV infection, as measured by a ≥4-fold increase in HI titer from June through August or August through November.

Study participants were monitored for school absences because of fever during the period associated with peak dengue transmission (June–November). Those participants with fever or history of fever in the prior 7 days were further evaluated with collection of acute and convalescent (>15 day) blood samples. DENV infection was identified by virus isolation and/or reverse-transcriptase polymerase chain reaction (RT-PCR) of acute serum samples. The infecting DV serotype was identified from acute-phase serum samples by use of a serotype-specific RT-PCR with use of previously published methods [12]. Virus isolation in *Toxorhnychites splendens* mosquitoes was performed with plasma samples from all symptomatic patients with use of methods described elsewhere [13]. Serologic examination to determine primary or secondary infection used dengue IgG/IgM enzyme-linked immunosorbent assay (ELISA) and HI assays against all 4 dengue serotypes [14]. All donors during symptomatic illness had their blood samples tested by both PCR and virus isolation.

A case was considered to be symptomatic if they had a school absence with a fever or history of fever and serologic findings consistent with acute dengue infection. Subclinical DENV infection was defined as a 4-fold increase in HI antibody against any DENV serotype between 2 sequential serum samples obtained during the surveillance months (June, August, or November) without a febrile illness identified during active surveillance during the period that seroconversion occurred. Serum samples were tested concurrently for Japanese encephalitis virus (JEV)-specific HI antibody to exclude JEV infection and antibody cross-reactivity as a cause for an increase in dengue antibody.

All laboratory-confirmed virus infections were characterized according to World Health Organization guidelines for dengue fever and DHF [15]. Informed consent was obtained from all patients or their parents, and these studies were approved by the institutional review boards at the University of Massachusetts Medical School; the Ministry of Public Health, Thailand; and the Human Subjects Research Review Board for the Commanding

General of the US Army Medical Research and Material Command.

From the larger cohort, we identified a group of patients who, by serologic testing, were known to have developed secondary DENV infections in 1998. From this group, 33 samples were randomly selected for analysis. The persons selected represented 6 of the 12 Kamphaeng Phet schools that participated in the study. Of the samples tested, 4 were eliminated from the analysis because of inadequate cell events (<50,000 events/tube). Two patients were excluded because the pre-illness serologic findings showed no detectable (titer, <1:10) neutralizing antibodies to any of the 4 DENV serotypes; one of these patients had a neutralizing antibody titer of 1:35 to JEV, and the other had no detectable neutralizing antibody to JEV. We compared 10 samples from children who developed symptomatic infection with 17 samples from children who had subclinical infection.

Antigen Preparation and Controls

Inactivated DENV-infected Vero cell lysates were prepared as described elsewhere [16]. Control antigens were prepared in a similar manner with use of uninfected Vero cells. The following viruses were used: DENV-1 Hawaii, DENV-2 New Guinea C, DENV-3 CH53489, and DENV-4 814669. Control antigen was a lysate of uninfected Vero cells. Phorbol Myristate Acetate at 20 μg/mL and ionomycin at 100 μg/mL were added as a positive control. A further negative control included cells incubated in media alone. All assays used a single lot of antigens, and each lot was tested for reactivity with monotypic DENV-immune donors [16].

Cytokine Flow Cytometry

Laboratory assays and analysis of flow cytometry data was done under code, with the investigator blinded to the clinical information. With use of previously established methods [16], PBMCs were stimulated with antigen at an antigen dilution of 1:20 for 18 h, at which time Brefeldin A was added; surface and intracellular cytokine staining was performed 6 h later. Cell phenotype was determined by the following surface stains: $CD3^+$ Pacific Blue, $CD8^+$ PERCP-Cy5.5, and $CD 14^+$ & $CD 19^+$ PE-CY7 (BD Biosciences) and $CD4^+$ Alexa 700 (eBiosciences). Cell viability was measured using the surface stain Live-Dead Aqua (Invitrogen). Intracellular cytokine production was measured using the following stains: TNF-α APC (BD Biosciences), IFN-γ APC-Alexa 750 (Caltag Laboratories), and IL-2 PE (eBiosciences). Data were acquired using FACS - ARIA flow cytometer. Positive responses were measured by subtracting the background percentage of cytokine-positive T cells in the control antigen tube; those tubes with an equal or smaller percentage of cells than that of the control antigen were considered to have no response. The data were analyzed using FlowJo, version 6.2.4 (Tree Star). Live-Dead$^-$ $CD3^+$ $CD14/19^-$ cells (both $CD4^+$ and $CD8^+$) were gated and analyzed for cytokine production.

Data Analysis

All data were analyzed using Stata software. Comparison of median frequencies of DENV-specific cytokine-producing $CD4^+$ and $CD8^+$ T cells between symptomatic and subclinical infection groups was done using the Wilcoxon rank sum test. The number of patients with detectable (above negative control) DENV-specific T cell cytokine responses between the symptomatic and subclinical groups for each DENV serotype was compared using Fisher's exact test.

RESULTS

Cohort Characteristics

Table 1 shows the characteristics of both groups defined on the basis of the clinical outcome during secondary infection: persons who experienced a febrile illness (ie, symptomatic group) and persons who had serologic evidence of infection without a recognized febrile illness (ie, subclinical group). Of the 10 patients in the symptomatic group, only 1 was hospitalized (this patient did not meet criteria for DHF), 6 had RT-PCR positive for DENV (5 with DENV-3 and 1 with DENV-1); the virus isolation results matched these findings.

DENV-Specific $CD4^+$ T Cell Cytokine Responses

Figure 2 shows the frequency of DENV-specific cytokine-producing cells (TNFα, IFNγ, and IL-2) in response to stimulation with each of the 4 DENV antigens in the PBMCs collected before the index secondary DENV infection. Regardless of clinical outcome, the majority of patients tested displayed a higher frequency of TNFα-producing cells after antigen stimulation, regardless of serotype, whereas the frequencies of IFNγ and IL-2 producing cells were much smaller for both groups.

The gating strategy is shown in Figure 1. Overall (ie, across serotypes and cytokines), the frequencies of DENV-specific cytokine-producing $CD4^+$ T cells were higher in the subclinical group than in the symptomatic group. The percentage of IL-2–producing cells was higher in the subclinical group after in vitro stimulation with DENV-1, DENV-2, and DENV-4 antigens, although the finding was statistically significant for only DENV-4 ($P = .01$). The percentage of IFNγ-producing cells responding to DENV-4 antigen was significantly higher in the subclinical group, as was the percentage of TNFα-producing cells responding to DENV-1 antigen.

The number of patients who showed measurable $CD4^+$ T cell cytokine responses to DENV antigen stimulation is shown in Table 2. For individual serotypes, there was a significantly higher proportion of subclinical IL-2 and IFNγ responders after stimulation with DENV-4. An additional serotype-specific association among responders was also seen in TNFα (to DENV-2), although this finding did not reach statistical significance.

Table 1. Serological Characteristics of the Study Population

Group	Subject	Plaque Reduction Neutralization Titers (PRNT50)					Acute Illness		
		Den 1	Den 2	Den 3	Den 4	JE	PCR +	Viral Culture	Den IgM/IgG
Symptomatic									
	105	3443	7456	1445	162	1	No	-	<1
	230	946	441	2657	210	323	No	-	<1
	247	7925	129	8232	1	17	No	-	<1
	592	1	152	1	1	1	Yes	D1	<1
	1136	745	21	176	56	1	Yes	D3	<1
	1250	>10240	471	1962	1	71	Yes	D3	<1
	1285[a]	1	299	150	1	1	Yes	D3	<1
	1307	211	898	3015	1	79	Yes	D3	<1
	1413	1	1	14	1	1	No	-	<1
	1484	1	377	1	1	21	Yes	D3	<1
Subclinical									
	143	1081	482	979	51	1			
	177	545	1030	219	1	1			
	209	562	110	452	112	64			
	431	130	478	71	53	1			
	585	4456	715	2619	1	1			
	628	2129	1	1674	1	1			
	637	1107	686	2226	1	42			
	963	>10240	211	465	15	1			
	982	2031	1	90	1	1			
	1281	636	332	2344	1	80			
	1294	1	1013	1	1	1			
	1327	364	435	210	1	1			
	1342	879	321	630	1	1			
	1374	2658	441	451	11	42			
	1402	1	261	1	1	53			
	1557	1036	191	4598	1	1			
	1577	16	906	124	1	1			

NOTE. The PRNT50 data refer to serum taken from all subjects as part of routine serologic surveillance in January 1998. The latter three columns refer to laboratory data taken at the time of acute illness. A PRNT50 value of 1 indicates an undetectable antibody response. An IgM/IgG ratio of <1.8 indicates secondary infection, while a ratio >1.8 indicates primary infection. The PCR, Viral culture, and DENV IgM data were unavailable for the subclinical group as these are routine tests during symptomatic infection.

[a] Hospitalized; did not meet WHO criteria for DHF.

DENV-Specific CD8^{+} T Cell Cytokine Responses

Figure 3 shows the frequency of DENV-specific cytokine-producing cells (TNFα, IFNγ, and IL-2) in response to stimulation with each of the 4 DENV antigens in the PBMCs collected before the index secondary DENV infection. Similar to the CD4^{+} T cell responses, stimulation with DENV antigens yielded a much higher frequency of TNFα-positive CD8^{+} T cells than for either IFNγ or IL-2.

As seen in Figure 3, the subclinical group again showed overall higher median frequencies of cytokine-producing cells after stimulation with DENV antigens. As with the CD4^{+} T cells, TNFα production was higher in the subclinical group after stimulation with DENV-1 antigen alone, whereas IFNγ production was higher in the subclinical group after stimulation with DENV-4 antigen ($P < .05$). No statistically significant differences in the pre-infection CD8^{+} T cell characteristics

between the symptomatic and subclinical groups were found with respect to IL-2 production.

The number of patients who generated CD8^{+} T cell cytokine responses after stimulation with DENV antigens is shown in Table 3. There was a significantly higher proportion of subclinical TNFα responders after stimulation with DENV-1 antigen ($P = .01$), in addition to a higher proportion of subclinical IFNγ responders to DENV-4, but this was not statistically significant ($P = .06$). No serotype-specific differences could be found with respect to IL-2.

Correlation Between Production of TNFα, IFNγ, and IL-2 at the Single-Cell Level

Because of the hypothesized importance of polyfunctional T cells in determining the outcome of the memory T cell response to virus infection, we examined the production of each
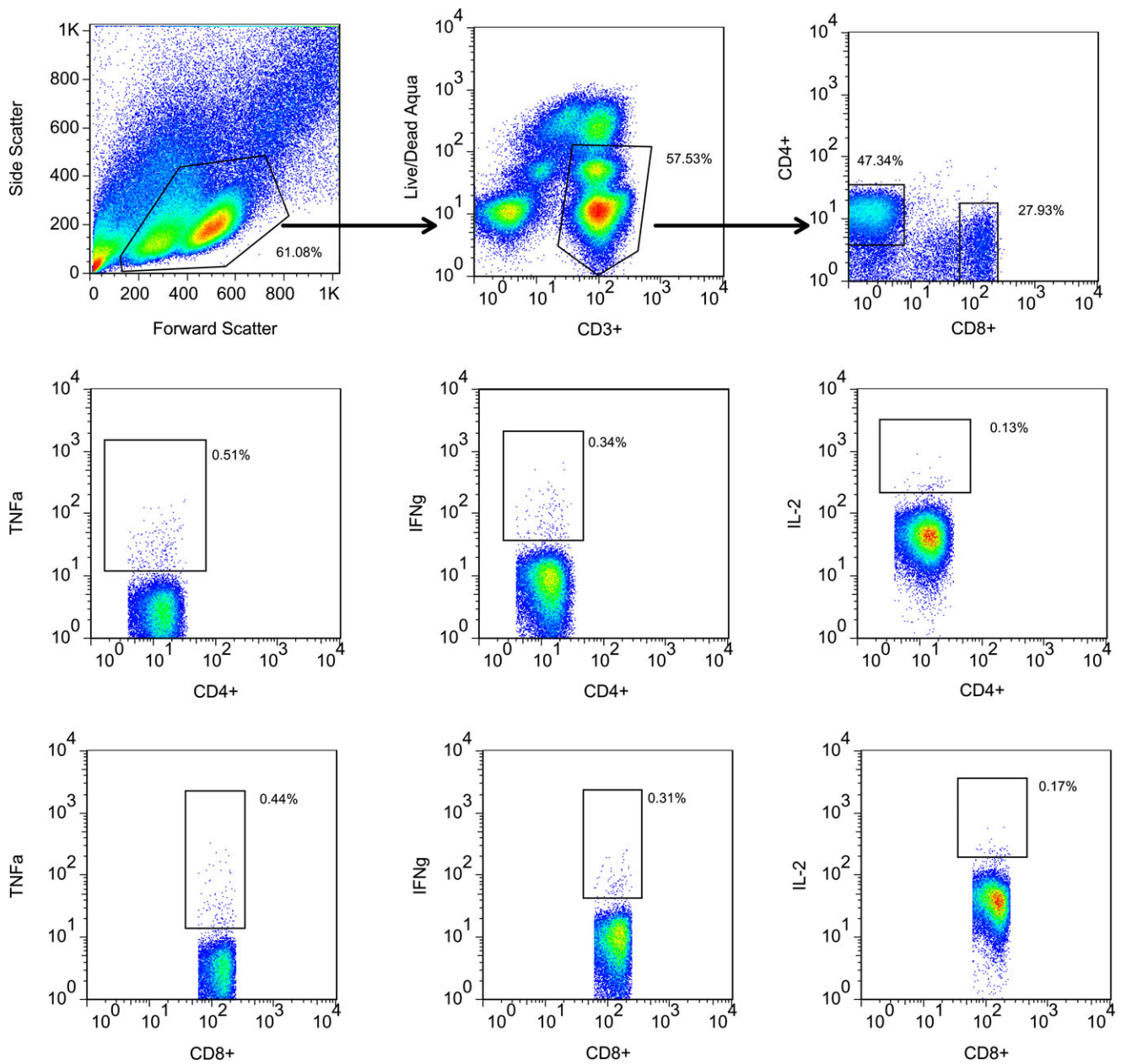
Figure 1. Gating strategy. Shown in the top row are gated lymphocytes in forward versus side scatter *(left)*; CD3$^+$, live/dead negative gated cells *(center)*; and CD4$^+$ or CD8$^+$ gated cells *(right)*. In the middle row are CD4$^+$ cells producing TNFα *(left)*, IFNγ *(center)*, or IL-2 *(right)*; the bottom row shows the same arrangement for CD8$^+$ cells. The frequencies of cytokine-producing cells were determined by subtraction of the background percentage of cytokine-producing antigen cells in the control antigen tube. This figure represents the PBMC responses from a patient with subclinical secondary infection; the antigen used to stimulate PBMCs here were derived from DENV-3.

of the 3 cytokines (TNFα, IFNγ, and IL-2) at the single cell level among DENV-specific T cell populations. After DENV antigen stimulation, the majority of responders generated monofunctional rather than polyfunctional cells. Only 2 donors, both in the subclinical group, had significant numbers of polyfunctional cells (Figure 4). In addition, there was no statistically significant association between production of any 2 cytokines after antigen stimulation, regardless of serotype (data not shown). This was true in both the CD4$^+$ and CD8$^+$ T cell populations.

DISCUSSION

To our knowledge, this is the first article to describe the host immune response to DENV infection in relation to subclinical secondary infection and to do so using pre-illness samples collected as part of a prospective study. We report increased frequencies of DENV-specific, cytokine-producing CD4$^+$ and CD8$^+$ cells among schoolchildren who subsequently experienced subclinical, compared with symptomatic secondary
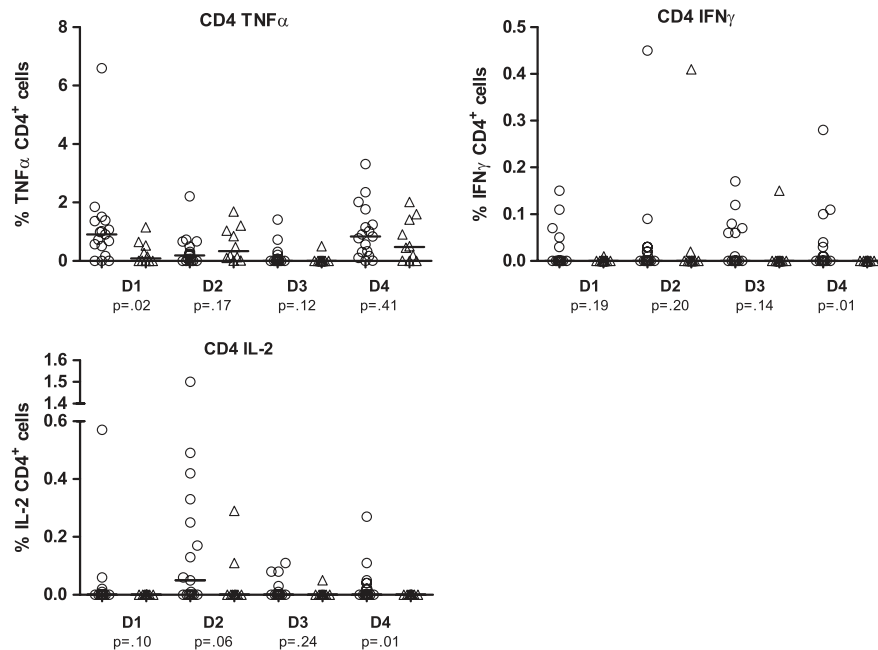
Figure 2. CD4^{+} intracellular cytokine production. TNFα, IFNγ, and IL-2 production was measured in response to antigen stimulation with 4 dengue serotypes of presecondary infection PBMC from 27 Thai schoolchildren. Comparison groups are 10 children *(triangles)* who developed symptomatic infection and 17 children *(circles)* who had subclinical infection established retrospectively through dengue IgG ELISA and HI titers. Median percentage of cytokine-producing cells and *P* values to detect significant median differences using the Wilcoxon rank sum test are shown.

DENV infections. Significantly higher frequencies with regard to IL-2 production were seen in the subclinical group after stimulation with DENV-4 antigen and higher frequencies after stimulation with DENV-1 and DENV-2, although these latter findings were not statistically significant. Differences between these subclinical and symptomatic groups in frequencies of DENV-specific IFNγ- and TNFα-producing CD4^{+} and CD8^{+} T cells was also present, with a significantly higher frequency of TNFα-producing T cells seen in the subclinical group after

stimulation with DENV-1 antigen and a higher frequency of IFNγ-producing T cells after stimulation with DENV-4 antigen. We also noted a higher likelihood of being a responder in the subclinical group, particularly with respect to IFNγ and IL-2 production, although the small sample size of the study precludes making definitive conclusions.

The pattern of T cell responses observed suggests a protective role for heterologous T cell responses through antiviral cytokine (ie, IFNγ and IL-2) production. Other studies indicated that

Table 2. Proportions of Subjects with Detectable Dengue-specific CD4^{+} T cell Cytokine Production from Presecondary Infection PBMC for Subclinical and Symptomatic Groups

		No. of responders: no. of nonresponders		
Cytokine	Antigen	SubclinicalSamples	Symptomatic Samples	P (by Fisher's exact test)
TNFα	D1	14:3	6:4	.36 (NS)
	D2	11:6	10:0	**.06**
	D3	7:10	1:9	.19 (NS)
	D4	16:1	8:2	.54 (NS)
IFNγ	D1	5:12	1:9	.36 (NS)
	D2	8:9	2:8	.23 (NS)
	D3	7:10	1:9	.19 (NS)
	D4	8:9	0:10	**.01**
IL-2	D1	4:13	0:10	.26 (NS)
	D2	10:7	2:8	.11(NS)
	D3	5:12	1:9	.36 (NS)
	D4	8:9	0:10	**.01**

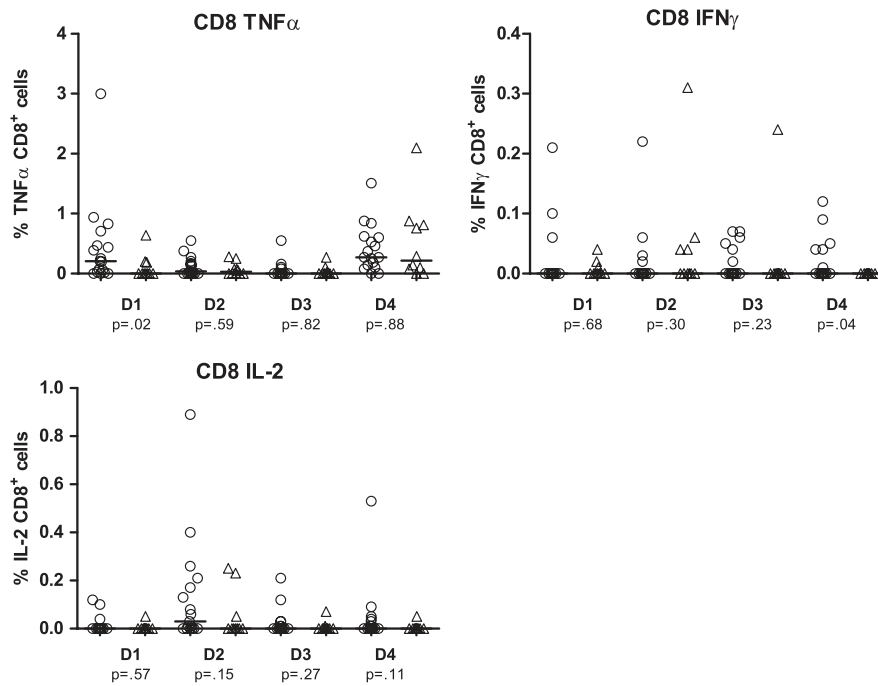**NOTE.** NS, not statistically significant difference in proportions.

Figure 3. CD8+ intracellular cytokine production. TNFα, IFNγ, and IL-2 production was measured in response to antigen stimulation with 4 dengue serotypes of presecondary infection PBMCs from 27 Thai schoolchildren. Comparison groups are 10 children *(triangles)* who developed symptomatic infection and 17 children *(circles)* who had subclinical infection established retrospectively through dengue IgG ELISA and HI titers. Median percentage of cytokine-producing cells and *P* values to detect significant median differences using the Wilcoxon rank sum test are shown.

pro-inflammatory cytokines, such as TNFα, generated in the heterologous response could have a harmful effect; in a previous study based on this same prospective cohort (albeit, involving a different group of children), our group compared in vitro cytokine secretion after antigen stimulation in PBMCs collected before secondary infection between children who were hospitalized and those who were not hospitalized during the subsequent secondary DENV infection. We reported that the

only TNFα responses were among children who were hospitalized [17]. That study did not include a separate subclinical infection group. In addition, the previous study measured cytokine secretion over a 6-day period after antigen stimulation. The longer assay involves in vitro expansion of memory T cells and measures total cytokine secretion in the culture supernatants rather than the frequency of cytokine-producing cells and may reflect different aspects of the T cell functional response

Table 3. Proportions of Patients with Detectable Dengue-specific CD8+ T cell Cytokine Production from Presecondary Infection PBMCs for Subclinical and Symptomatic Groups

| | | No. of responders: no. of nonresponders | | |
Cytokine	Antigen	Subclinical samples	Symptomatic samples	P (by Fisher's exact test)
TNFα	D1	14:3	3:7	**.01**
	D2	11:6	6:4	>.99 (NS)
	D3	4:13	3:7	>.99 (NS)
	D4	15:2	8:2	.59 (NS)
IFNγ	D1	3:14	3:7	.64 (NS)
	D2	4:13	4:6	.41 (NS)
	D3	6:11	1:9	.20 (NS)
	D4	6:11	0:10	**.06**
IL-2	D1	3:14	1:9	>.99 (NS)
	D2	11:6	3:7	.12 (NS)
	D3	7:10	2:8	.41 (NS)
	D4	7:10	1:9	.19 (NS)

NOTE. NS, not statistically significant difference in proportions.
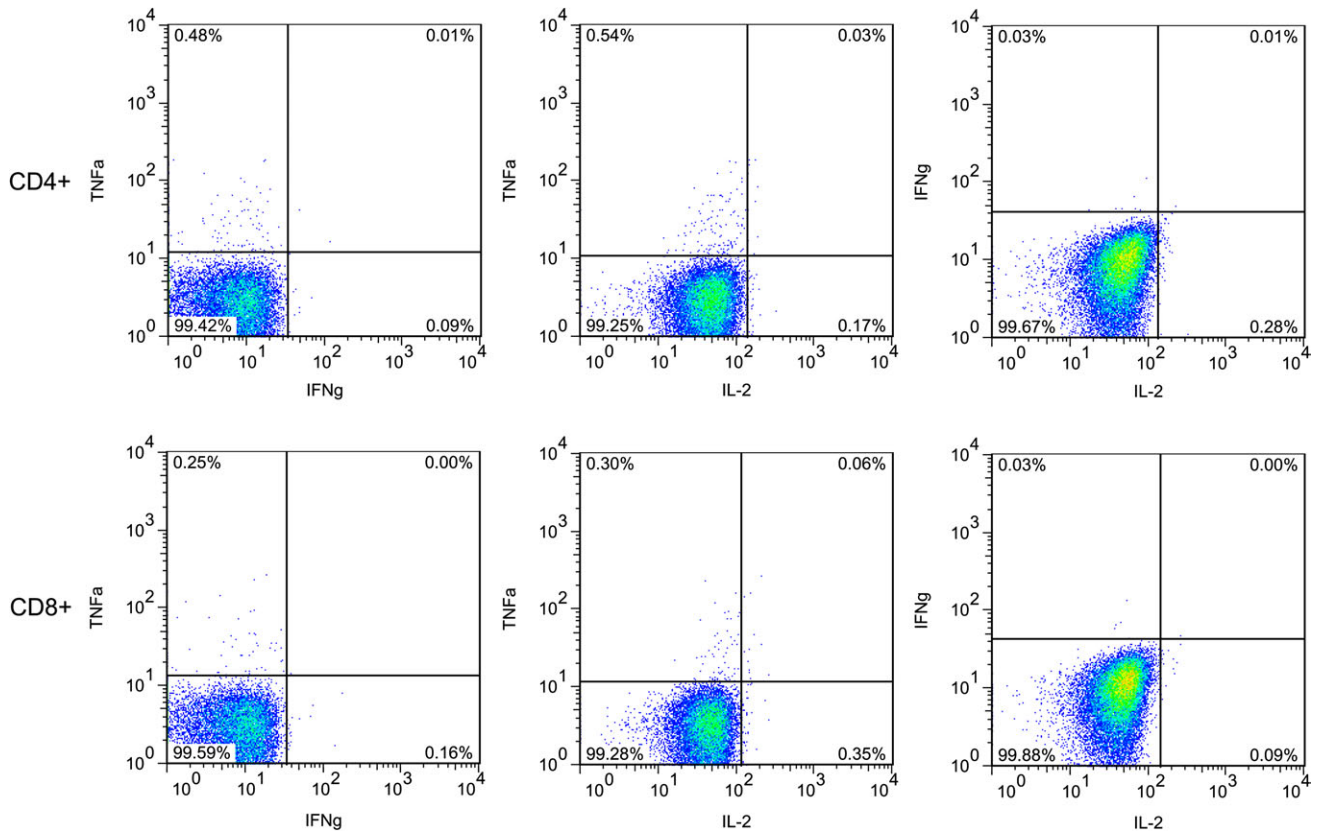
Figure 4. Lack of polyfunctionality in cytokine production following antigen stimulation. Twenty-five of the 27 donors had T cells that produced only 1 cytokine *(upper left and lower right quadrants)* but did not have cells that simultaneously produced 2 cytokines *(upper right quadrants)*. The top row represents CD4+ cells, and the bottom row represents CD8+ cells. At left is TNFα (y-axis) versus IFNγ (x-axis); the center shows TNFα versus IL-2; the right shows IFNγ versus IL-2.

than the overnight ex vivo flow cytometry assay. However, there was a tendency for increased IFNγ responses in the group with less severe symptoms, as seen in the present study.

These differences in relative production of cytokines are consistent with other studies of DENV-specific memory T cells. We reported higher frequencies of TNFα- producing serotype–cross-reactive DENV-specific memory CD4$^+$ T cells, compared with IFNγ-producing cells in PBMCs from recipients of candidate monovalent, live DENV vaccines [18]. By contrast, we also found a less consistent hierarchy of responses among individual DENV epitope–specific CD8$^+$ T cells after primary infection [19], and Imrie et al [20] showed altered cytokine responses at the clonal level among CD8$^+$ cells in Pacific Islanders after DENV-1 infection with a hierarchy of IFNγ, TNFα, and IL-2. In these studies, however, no associations could be drawn with regard to symptoms after subsequent secondary infection.

We cannot completely exclude the possibilities that the subclinical group had more recently experienced their previous DENV infection or had experienced 2 (or more) prior DENV infections, explaining both a higher frequency of DENV-specific antiviral cytokine-producing T cells and less symptomatic secondary infection. The narrow age range in the cohort and the

absence of statistically significant differences between groups in the pre-illness PRNT data (Table 1) make this interpretation less likely, however, and our data still point to a relationship between the frequency of cytokine-producing T cells and subclinical infection. In addition to the immune response profile that we measured, a multiplicity of factors could influence the host immune response during secondary infection, resulting in the presence or absence of symptoms. For instance, we reported that certain HLA alleles were associated with disease severity in this population [21]. Although that study examined only symptomatic DENV-infected patients, it suggests that host genetic factors can be either protective or harmful in determining the presence or absence of symptoms in secondary infection. Other factors, including the immunologic similarities of DENV epitopes, the virus inoculum and/or the magnitude of the viremia during primary or secondary infection, the virulence of the strain [22], and dysregulation of the complement system [23], could contribute to differences in disease severity.

Statistically significant differences in cytokine-producing T cell frequencies after DENV-3 antigen stimulation were not prominent, although the majority of detectable infections in the symptomatic group involved DENV-3. Subgroup analysis of the

patients with known DENV-3 infections did not provide further insights, largely because of small sample size. Although we cannot know with certainty the infecting serotype in the subclinical cases, our study and other data from Kamphaeng Phet province in that year indicate that DENV-3 was the predominant circulating serotype [24]. The response to other DENV serotypes detected in the flow cytometry assay, reflecting T cell responses generated by the earlier primary DENV infection, includes serotype–cross-reactive T cells that may be capable of blunting the severity of secondary infection with DENV-3.

Limitations of the current study include the use of an inactivated antigen preparation (Vero cell lysates) rather than overlapping peptides or known DENV epitopes. Although the drawbacks of this approach are that we could not identify specific immunodominant epitopes and that stimulation of CD8$^+$ T cells by the inactivated antigen is probably suboptimal, the use of DENV antigen allowed us to evaluate the immunologic response to the entire proteome of all 4 DENV serotypes using the small number of PBMCs available from these pediatric patients. Another limitation is that the symptomatic group represented only mild infection. A separate group of patients with DHF would permit further definition of immunologic associations with disease. In addition, we only studied the production of IFNγ, TNFα, and IL-2. The production of other cytokines, such as IL-4 and IL-18, which have been implicated in dengue disease severity, might also be of interest [25, 26].

In summary, we believe that our study supports the model that cytokine imbalance created by stimulation of cross-reactive T cells contributes to dengue disease severity and that ours is the first study to evaluate the predictive value of cytokine flow cytometry among children who later experienced secondary DENV infection. However, the relationship between cross-reactive T cells and the role played by heterologous antibodies has yet to be examined. Another important line of inquiry would be to compare the findings of this study to those for genetically distinct human populations, such as in countries in the Western Hemisphere where dengue is endemic.

Establishing the relationships between antibody and T cell responses to dengue infection will be of particular value as vaccine development reaches fruition. The need to identify measurable correlates of protective immunity and to find immunologic markers predictive of such protection is critical. One recent study evaluated cell-mediated immunity during a phase 1 clinical trial of a chimeric tetravalent dengue vaccine [27], although data from phase 2 and 3 trials has yet to be analyzed. Because of the unique challenges in developing a tetravalent vaccine for dengue, witnessed by the difficulties encountered by vaccine manufacturers until now [28], a thorough understanding of the cellular immunology of secondary infection will be invaluable to evaluate the effectiveness of the several candidate vaccines currently in development.

References

1. Kroeger A, Nathan M, Hombach J. Dengue. Nat Rev Microbiol 2004; 2:360–1.
2. Dengue fever climbs the social ladder. Nature 2007; 448:734–5.
3. Sabin AB. Research on dengue during World War II. Am J Trop Med Hyg 1952; 1:30–50.
4. Okuno Y, Fukunaga T, Srisupaluck S, Kasemsarn P, Dharakul C, Sangkawibha N. Serological and virological studies on patients with dengue hemorrhagic fever (DHF) in Chanthaburi province, Thailand. I. Serological studies on paired sera from DHF patients by neutralization (N), hemagglutination inhibition (HI) and staining tests. Biken J 1980; 23:113–21.
5. Sangkawibha N, Rojanasuphot S, Ahandrik S, et al. Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak. Am J Epidemiol 1984; 120:653–69.
6. Graham RR, Juffrie M, Tan R, et al. A prospective seroepidemiologic study on dengue in children four to nine years of age in Yogyakarta, Indonesia I. studies in 1995-1996. Am J Trop Med Hyg 1999; 61:412–9.
7. Guzman MG, Kouri G. Dengue and dengue hemorrhagic fever in the Americas: lessons and challenges. J Clin Virol 2003; 27:1–13.
8. Kliks SC, Nisalak A, Brandt WE, Wahl L, Burke DS. Antibody-dependent enhancement of dengue virus growth in human monocytes as a risk factor for dengue hemorrhagic fever. Am J Trop Med Hyg 1989; 40:444–51.
9. Kurane I, Rothman AL, Livingston PG, et al. Immunopathologic mechanisms of dengue hemorrhagic fever and dengue shock syndrome. Arch Virol Suppl 1994; 9:59–64.
10. Rothman AL, Ennis FA. Immunopathogenesis of Dengue hemorrhagic fever. Virology 1999; 257:1–6.
11. Endy TP, Chunsuttiwat S, Nisalak A, et al. Epidemiology of inapparent and symptomatic acute dengue virus infection: a prospective study of primary school children in Kamphaeng Phet, Thailand. Am J Epidemiol 2002; 156:40–51.
12. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J Clin Microbiol 1992; 30:545–51.
13. Vaughn DW, Green S, Kalayanarooj S, et al. Dengue in the early febrile phase: viremia and antibody responses. J Infect Dis 1997; 176:322–30.
14. Innis BL. Antibody responses to dengue virus infection. In Gubler DJ, Kuno G, eds: Dengue and dengue hemorrhagic fever. New York: CAB International, 1997; 221–43.

15. Dengue: Guidelines for Diagnosis, Treatment, Prevention Control. World Health Organization, 2009.
16. Mangada MM, Ennis FA, Rothman AL. Quantitation of dengue virus specific CD4+ T cells by intracellular cytokine staining. J Immunol Methods 2004; 284:89–97.
17. Mangada MM, Endy TP, Nisalak A, et al. Dengue-specific T cell responses in peripheral blood mononuclear cells obtained prior to secondary dengue virus infections in Thai schoolchildren. J Infect Dis 2002; 185:1697–703.
18. Mangada MM, Rothman AL. Altered cytokine responses of dengue-specific CD4+ T cells to heterologous serotypes. J Immunol 2005; 175:2676–83.
19. Bashyam HS, Green S, Rothman AL. Dengue virus-reactive CD8+ T cells display quantitative and qualitative differences in their response to variant epitopes of heterologous viral serotypes. J Immunol 2006; 176:2817–24.
20. Imrie A, Meeks J, Gurary A, et al. Differential functional avidity of dengue virus-specific T-cell clones for variant peptides representing heterologous and previously encountered serotypes. J Virol 2007; 81:10081–91.
21. Stephens HA, Klaythong R, Sirikong M, et al. HLA-A and -B allele associations with secondary dengue virus infections correlate with disease severity and the infecting viral serotype in ethnic Thais. Issue Antigens 2002; 60:309–18.
22. Rico-Hesse R. Microevolution and virulence of dengue viruses. Adv Virus Res 2003; 59:315–41.
23. Nascimento EJ, Silva AM, Cordeiro MT, et al. Alternative complement pathway deregulation is correlated with dengue severity. PLoS One 2009; 4:e6782.
24. Endy TP, Nisalak A, Chunsuttitwat S, et al. Relationship of preexisting dengue virus (DV) neutralizing antibody levels to viremia and severity of disease in a prospective cohort study of DV infection in Thailand. J Infect Dis 2004; 189:990–1000.
25. Bozza FA, Cruz OG, Zagne SM, et al. Multiplex cytokine profile from dengue patients: MIP-1beta and IFN-gamma as predictive factors for severity. BMC Infect Dis 2008; 8:86.
26. Mustafa AS, Elbishbishi EA, Agarwal R, Chaturvedi UC. Elevated levels of interleukin-13 and IL-18 in patients with dengue hemorrhagic fever. FEMS Immunol Med Microbiol 2001; 30:229–33.
27. Guy B, Nougarede N, Begue S, et al. Cell-mediated immunity induced by chimeric tetravalent dengue vaccine in naive or flavivirus-primed subjects. Vaccine 2008; 26:5712–21.
28. Hatch S, Mathew A, Rothman A. Dengue vaccine: opportunities and challenges. IDrugs 2008; 11:42–5.