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Dengue Virus-Reactive CD8$^+$ T Cells Display Quantitative and Qualitative Differences in Their Response to Variant Epitopes of Heterologous Viral Serotypes

Hema S. Bashyam, Sharone Green, and Alan L. Rothman

Reactivation of serotype cross-reactive CD8$^+$ memory T lymphocytes is thought to contribute to the immunopathogenesis of dengue disease during secondary infection by a heterologous serotype. Using cytokine flow cytometry, we have defined four novel HLA-A*02-restricted dengue viral epitopes recognized by up to 1.5% of circulating CD8$^+$ T cells in four donors after primary vaccination. All four donors had the highest cytokine response to the epitope NS4b 2353. We also studied the effect of sequence differences in heterologous dengue serotypes on dengue-reactive CD8$^+$ memory T cell cytokine and proliferative responses. The D3 variant of a different NS4b epitope 2423 and the D2 variant of the NS4a epitope 2148 induced the largest cytokine response, compared with their respective heterologous sequences in all donors regardless of the primary vaccination serotype. Stimulation with variant peptides also altered the relative frequencies of the various subsets of cells that expressed IFN-γ, TNF-α, MIP-1β, and combinations of these cytokines. These results indicate that the prior infection history of the individual as well as the serotypes of the primary and heterologous secondary viruses influence the nature of the secondary response. These differences in the effector functions of serotype cross-reactive memory T cells induced by heterologous variant epitopes, which are both quantitative and qualitative, may contribute to the clinical outcome of secondary dengue infection. The Journal of Immunology, 2006, 176: 2817–2824.

Several recent studies have highlighted the importance of T cell cross-reactivity in mediating immune responses during infections with heterologous Ags. In these studies, reactivation of the cross-reactive T cell component of the memory pool by unrelated viruses resulted in altered T cell epitope hierarchies (1) and reshaped T cell repertoires (2). These changes influenced the ultimate outcome of sequential infection by improving protective immunity (3) or, in some instances, by enhancing immunopathology (4). The mechanisms of heterologous immunity that may lead to a negative outcome have been inadequately studied in humans.

Dengue virus infections offer a clear paradigm for investigating the role of cross-reactive memory T cells during an immune response to sequential infections with heterologous viruses. The four natural variants (serotypes) of dengue virus (1–4) can cause an acute infection in humans that presents clinically either as classical dengue fever (DF) (5, 6). Primary infection with any of the four serotypes results in lifelong immunity to the same serotype but leaves the individual susceptible to sequential secondary infections by heterologous serotypes (7). Epidemiological studies in Cuba and Southeast Asia have shown a strong association between secondary dengue infection and DHF (8, 9). These data indicate that previous immunity to dengue may be a risk factor in the development of severe dengue disease (10, 11). The mechanisms proposed for this effect involve serotype cross-reactive, non-neutralizing Abs, which can enhance infection, and serotype cross-reactive memory T lymphocytes.

We and others have postulated that serotype cross-reactive T cells in the memory repertoire are preferentially activated and expanded during secondary infection and contribute to DHF immunopathogenesis through an enhanced output of effector function (12, 13). In support of this hypothesis, CD8$^+$ T cells from patients with secondary dengue infection show higher binding to tetramers with heterologous epitope sequences than to tetramers with sequences of the secondary serotype, revealing the higher affinity of these cells for other presumably previously encountered dengue serotypes (13). Plasma levels of IFN-γ, TNF-α, soluble TNFRs (sTNFRs), sIL-2R, and sCD8 have been found to be higher in patients with DHF than in those individuals with DF (14–16). PBMC from patients with DHF have a higher percentage of CD69$^+$CD8$^+$ cells as well as a higher frequency of dengue epitope-specific CD8$^+$ lymphocytes, compared with PBMC from patients with DF (17, 18).

In vitro experiments, heterologous restimulation of dengue-immune PBMC induced only a partial activation of a subset of serotype cross-reactive cells specific for a dengue epitope. This epitope was characterized as a partial agonist in its heterologous form because it induced poor proliferation and IFN-γ production in a CTL clone while able to sensitize target cells for lysis (19). Consequently, we have hypothesized that the cross-reactive epitopes of the secondary heterologous serotypes may act as altered peptide ligands for dengue-specific cross-reactive CD8$^+$ T cells and effect changes in the overall functional response of this
population by varying T cell proliferation, cytokine/chemokine production, and cytotoxicity.

Several studies that examined the functional potential of human memory CD8\(^+\) T cells in systems such as HIV, CMV, and Epstein–Barr virus have reported a polyclonal population of Ag-specific cells that include subsets with a diverse array of partial and full effector functions (20–24). These subsets include cytokine-competent but cytolysis-defective cells, proliferating but cytokine-deficient cells, and cell populations that secrete a limited range of cytokines. This functional heterogeneity of the memory population suggests that the quality of the recall response will depend on which of these different subsets undergo selective expansion and dominate the secondary response.

The present study was designed to determine whether stimulation of dengue epitope-specific memory CD8\(^+\) T cells with variant sequences from heterologous serotypes quantitatively or qualitatively alter the range of their effector functions. We used PBMC from individuals immunized with experimental monovalent live-attenuated dengue vaccines. Four new HLA-A*02-restricted CD8\(^+\) T cell epitopes were identified, and the responses to the variants from each serotype were studied by staining for intracellular IFN-\(\gamma\), TNF-\(\alpha\), MIP-1\(\beta\), and analyzing proliferation by CFSE. Stimulation with epitope variants resulted in quantitative and qualitative changes in proliferative and cytokine responses. The presence of such epitope-specific cross-reactive cells within different (serotype-dependent) effector function potential during in vitro secondary response offers a mechanistic insight into in vivo immunopathogenesis during secondary dengue infection.

Materials and Methods

Human PBMC

Peripheral blood samples were obtained from four volunteers who had been immunized with experimental live-attenuated dengue vaccines (Table I) (25–28). A flavivirus-naive Massachusetts resident also was included in the study. PBMC were isolated by Ficoll-Hypaque and density gradient centrifugation. They were resuspended at 10\(^7\)/ml in RPMI 1640 containing 20% FBS and 10% DMSO and cryopreserved until use. HLA typing of these donors done at the tissue typing center at the University of Massachusetts Medical Center (Worcester, MA) identified all the donors as HLA-A*02.**

Peptides

The amino acid sequence of serotype 3 H87 strain (GenBank accession no. NC 001475) was initially chosen to generate a list of nonamer epitopes based on the peptide binding motif for HLA-A*0201 using the epitope prediction algorithm (available online at http://bimas.dkrct.nih.gov/molbio/hla.bind). Twenty-five sequences with the highest binding scores were chosen, and the peptides were synthesized at the University of Massachusetts Medical School Peptide Core Facility. The four identified epitopes were identical in D3 CH5348 (GenBank accession no. AF017733, non-structural; and GenBank accession no. M86733, structural). Variant peptides were synthesized based on the D1 45AZS (GenBank accession no. NC.001477), D2 NGC (GenBank accession no. M29095), and D4 814669 (GenBank accession no. AF326573).

Table I. Vaccination history and class I HLA alleles of study subjects

<table>
<thead>
<tr>
<th>Donors</th>
<th>Serotype and Vaccine Strain</th>
<th>Months after Vaccination PBMC Obtained</th>
<th>Class I HLA Type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>1</td>
<td>D1 45AZS</td>
<td>4, 8</td>
<td>2.11</td>
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<tr>
<td>2</td>
<td>D3 CH5348</td>
<td>168, 192</td>
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<tr>
<td>3</td>
<td>D2 16681</td>
<td>12</td>
<td>2.29</td>
</tr>
<tr>
<td>4</td>
<td>D2 16681</td>
<td>12</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Intracellular cytokine staining and flow cytometry

PBMC were thawed and washed with RPMI 1640 containing 10% heat-inactivated human AB serum. Cells (10\(^7\)) were stimulated with 10 \(\mu\)g/ml peptide and incubated at 37\(^\circ\)C for a total of 6 h with Golgi Plug (Brefeldin A; BD Pharmingen) added during the last 5 h. PBMC were washed with FACS buffer (2% FBS and 0.1% sodium azide in PBS) and incubated with 50 \(\mu\)g/ml mouse IgG for 15 min at 4\(^\circ\)C to block nonspecific binding to IgG Fc receptors. Cells were then stained for surface markers such as CD3 (PE-Cy7 Ab; Caltag Laboratories) and CD8 (PerCP Ab; BD Biosciences) at 4\(^\circ\)C for 30 min. After washing with FACS buffer, cells were fixed and permeabilized with the Cytofix/Cytoperm reagent (BD Pharmingen) for 20 min at 4\(^\circ\)C. This was followed by a wash in PermWash buffer (BD Pharmingen) and intracellular staining with Abs for CD69, TNF-\(\alpha\) (allophycocyanin), MIP-1\(\beta\) (PE) at 4\(^\circ\)C for 30 min. All Abs were obtained from BD Pharmingen. Data were acquired on a FACSaria machine (BD Biosciences) and analyzed using versions 4.2, 4.5, or 6.1 of FlowJo software (Tree Star). The lymphocyte gate included side-scatter and forward-scatter low populations that were selected for CD3\(^+\) and CD8\(^+\) cells. The number of events collected for each sample varied between 150,000 and 250,000, depending on the donor. The number of CD3\(^+\)CD8\(^+\) analyzed in each of the donors are as follows: donor 1, 20,000 \(\pm\) 2,000; donor 2, 27,000 \(\pm\) 2,000; donor 3, 44,000 \(\pm\) 3,000; donor 4, 60,000 \(\pm\) 3,000. Each experiment included isotype controls for each cytokine-Ab conjugate. Based on these, gates were first set on the medium-stimulated panel, and the same gating was then applied to all other samples within each donor.

CFSE staining and bulk culture of PBMC

PBMC were thawed and resuspended in RPMI 1640 medium at 10 \(\times\) 10\(^6\)/ml. Cells were stained with CFSE at 1 \(\mu\)g/ml for 25 min at 37\(^\circ\)C in the dark. Cells were then washed three times with cold RPMI 1640 medium containing 10% FBS, stimulated with 25 \(\mu\)g/ml peptide and 25 ng/ml IL-7, and incubated in the dark. IL-2 (50 U/ml) was added on day 3. A total of 3 \(\times\) 10\(^6\) bulk culture cells were mixed with 7 \(\times\) 10\(^6\) autologous B lymphoblastoid cell lines and restimulated with 10 \(\mu\)g/ml peptides on day 5 and day 14 and stained for surface markers (CD3 and CD8) and intracellular cytokines (IFN-\(\gamma\)-allophycocyanin, TNF-\(\alpha\)-AlexaFluor 610, MIP-1\(\beta\)-PE) as previously described.

Results

Identification of HLA-A*0201-restricted epitopes from PBMC of vaccinated individuals

To identify dengue virus-specific T cell epitopes for our study, we generated a panel of 25 9-mer peptides that fit the HLA-A*0201 binding motif and used these to stimulate PBMC from a dengue 3-immune HLA-A*02 donor. Frequencies of \(>0.1\%\) of CD69\(^+\) IFN-\(\gamma\)-producing cells were detected in the CD3\(^+\)CD8\(^+\) T cell population in response to stimulation by four of the peptides, with the highest response being 0.45% to the NS4b 2353 peptide (Fig. 1A). No frequencies of cells specific for NS4b 2353, NS4b 2423, and NS4a 2148 were higher than the frequency of cells specific for two previously identified HLA-B*62 epitopes in this donor (19). Responses to these four peptides also were detected in three other donors who were vaccinated with other dengue serotypes (Table II). No responses were detected in a HLA-A*02-negative dengue-immune donor or in a HLA-A*02-positive flavivirus-naive donor (data not shown).

In the dengue 3-immune donor, all four peptides induced a TNF-\(\alpha\) response that was higher than the corresponding IFN-\(\gamma\) response, suggesting that the epitope-specific population was comprised of subpopulations of cells that secreted TNF-\(\alpha\) but not IFN-\(\gamma\). In addition to IFN-\(\gamma\), TNF-\(\alpha\), and CD69 up-regulation, three of the four peptides also induced proliferation responses as measured by CFSE dilution (Fig. 1B). Cells that underwent more than six divisions over 5 days ranged from 5 to 16% of CD3\(^+\)CD8\(^+\) T cells remaining in the culture, which indicates that not all cytokine-producing cells respond by proliferating.
Altered patterns of IFN-γ, TNF-α, and MIP-1β coexpression following heterologous stimulation by variant epitopes

Because of variation in amino acid sequences between the four dengue serotypes (Table III) and the evidence from initial studies that vaccinated individuals responded to heterologous serotypes, we next investigated the range of cytokine production among each epitope-specific T cell population and determining whether these expression profiles were skewed following stimulation with variant sequences of these epitopes from heterologous serotypes. Of the four epitopes, the E493 and the NS4b 2353 epitopes are closely conserved within all four serotypes, whereas the NS4a 2148 and the four epitopes, the E493 and the NS4b 2423 epitopes are closely conserved in the donor’s vaccination did not always induce the highest response. Although PBMC from donors 2 and 4 showed the highest cytokine response when stimulated with their homologous peptides, stimulation of PBMC from donors 1 and 3 with heterologous peptides induced higher cytokine responses. Changes also were seen in the pattern of cytokine expression in response to heterologous stimulation. In donor 1, the D3 variant induced 0.76% IFN-γ/MIP-1β and 0.37% MIP-1β+ cells, whereas the D2 and D4 variants skewed the response, with most of the responding cells producing MIP-1β alone. The skewing of the cytokine profile also was apparent in donor 4 where the prevalent populations in response to D2 peptide stimulation were TNF-α+, IFN-γ/TNF-α+, and IFN-γ/MIP-1β+ cells. In contrast, the prevalent populations in response to D3 peptide stimulation were IFN-γ+ and IFN-γ+TNF-α+ cells.

Triple staining for IFN-γ, TNF-α, and MIP-1β: variant peptides alter both the size of the cytokine response as well as the relative frequencies of the various heterogeneous cytokine subsets

Because the initial double-staining experiments revealed the existence of distinct subsets of epitope-specific cells that were either single-positive for each cytokine or double-positive for IFN-γ/TNF-α and IFN-γ/MIP-1β, we wanted to further define the heterogeneity of these populations by checking for coexpression of TNF-α and MIP-1β. An example of the gating analysis of staphylococcal enterotoxin B-stimulated samples stained simultaneously with IFN-γ, TNF-α, and MIP-1β is shown in Fig. 3. Seven different populations of cytokine-producing cells (IFN-γ+, TNF-α+,

Table II. Frequencies of IFNγ-secreting epitope-specific CD8+ T cells in PBMC of dengue-immune subjects

<table>
<thead>
<tr>
<th>Start Position</th>
<th>Protein</th>
<th>Serotype</th>
<th>Sequence</th>
<th>Scorea</th>
<th>% IFN-γ-Producing CD69+ Cellsb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>2353</td>
<td>NS4b</td>
<td>D3</td>
<td>VLLLVTHYA</td>
<td>171</td>
<td>0.64</td>
</tr>
<tr>
<td>2423</td>
<td>NS4b</td>
<td>D3</td>
<td>LLLMLRTSWA</td>
<td>171</td>
<td>0.47</td>
</tr>
<tr>
<td>2148</td>
<td>NS4a</td>
<td>D3</td>
<td>LLLGMLTLL</td>
<td>309</td>
<td>0.35</td>
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<tr>
<td>493</td>
<td>E</td>
<td>D2</td>
<td>FLDLPLPWM</td>
<td>497</td>
<td>0.19</td>
</tr>
</tbody>
</table>

a Scores indicate the predicted half-time of dissociation from class I HLA molecules.
b PBMC were first gated on CD3+ CD8+ lymphocytes.
c Donors (Table I).
d Data are representative of one of several independent experiments.

Table III. Sequences of epitopes and their heterologous variants

<table>
<thead>
<tr>
<th>Start Position</th>
<th>Protein (Residues)</th>
<th>Serotype</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>2353</td>
<td>NS4b (111–119)</td>
<td>D1</td>
<td>VLLLVTHYA</td>
</tr>
<tr>
<td>2423</td>
<td>NS4b (181–189)</td>
<td>D1</td>
<td>LLLMLRTSWA</td>
</tr>
<tr>
<td>2148</td>
<td>NS4a (56–64)</td>
<td>D1</td>
<td>LLLMLMLTLL</td>
</tr>
<tr>
<td>493</td>
<td>E (211–219)</td>
<td>D1</td>
<td>FLDLPLPWM</td>
</tr>
</tbody>
</table>

Cell division in CD3

FIGURE 1. Identification of four novel HLA-A*0201-restricted T cell epitopes. PBMC from donor 2 (D3 immune) were stimulated for 6 h and analyzed by FACS as described in Materials and Methods. A, Contour plots show intracellular staining for IFN-γ, TNF-α, and CD69 in the CD3+CD8+ lymphocyte population. B, PBMC from donor 2 were stained with 1μM CFSE and stimulated by peptides for 5 days. Panels represent cell division in CD3+CD8+T cells, which are further gated into cells that have divided two to five times and cells that have undergone more than five divisions.
MIP-1β⁺, IFN-γ⁺TNF-α⁺, IFN-γ⁺MIP-1β⁺, TNF-α⁺MIP-1β⁺, and IFN-γ⁺TNF-α⁺MIP-1β⁺) were identified (Fig. 3, D–I). Results of such analyses were highly reproducible. In one individual (donor 2) with large numbers of PBMC available, the analysis was repeated in three separate experiments, and in almost all cases, the SD was ≤0.03%; the pattern of responses across serotypes was consistent in all repeated experiments. However, not all of the epitopes and their variants induced all seven cytokine subsets (Fig. 4). For example, stimulation of D1-immune PBMC (donor 1) with the D3 NS4b 2353 resulted in all seven functional populations, with the IFN-γ⁺MIP-1β⁺ subset being the largest. In contrast, the D1 NS4b 2353 peptide induced IFN-γ⁺, TNF-α⁺, MIP-1β⁺, and a very small population of TNF-α⁺MIP-1β⁺ cells (Fig. 4A). Similarly, in donor 2, although the homologous D3 NS4b 2423 peptide induced six different subsets of cytokine-producing cells, stimulation of D3 immune PBMC with the other variants of this epitope only gave rise to mainly IFN-γ⁺ and MIP-1β⁺ cells (Fig. 4B). Thus, heterologous peptide stimulation changed not only the size of the response but also altered its qualitative nature.

Other donor-specific patterns of cytokine production were also observed. For example, in donor 2, except for D3 variants of the NS4b peptides and D2 NS4a 2148, the response to the other variants of all four epitopes are mediated either by IFN-γ⁺ or MIP-1β⁺ cells or both. In contrast, the responses to all variants of all the epitopes in donor 3 were mediated by all seven subsets and the frequency of cells producing MIP-1β were lower than the frequencies of cells producing IFN-γ and TNF-α for all epitope variants.

When we looked at total cytokine responses induced by each variant, we found that D3 NS4b 2423 and D2 NS4a 2148 variants induced the highest responses in all four donors irrespective of their primary immunization serotype (Fig. 4, B and C). However, the profile of the response to each variant differed in each donor. Thus, the heterologous response to D3 NS4b 2423 in donors 1, 3, and 4 were dominated by MIP-1β or TNF-α, while the homologous response to this peptide in donor 2 was dominated by MIP-1β and IFN-γ. Although the heterologous response to D2 NS4a 2148 in donors 1 and 2 were dominated by IFN-γ-producing cells, the
homologous response in donors 3 and 4 was represented equally by IFN-γ/TNF-α and IFN-γ/MIP-1β populations, respectively.

The total numbers of CD8+ T cells responding functionally to all four epitopes ranged from 0.65 to 4% with cells responding to the variants of the NS4b 2353 epitope dominating the overall response in all four donors (Fig. 4e). As seen with D3 NS4b 2343 and D2 NS4a 2148, the same epitope variants induced the highest quantitative response in all donors, but the response in each donor differed in its qualitative nature. Higher responses to heterologous variants were more common than the responses to homologous peptides.

FIGURE 4. Quantitative and qualitative alterations in functional response following heterologous peptide stimulation. Triple cytokine stainings was performed on PBMC from all four donors stimulated with variants of all four epitopes. Each graph displays the responses to the four variants of the peptide indicated at the top of the column of the PBMC from the donor listed on the left. The height of each bar shows the total frequency of all CD3+CD8+ T cells responding to that peptide. Each bar also is divided into three bars that show the combined frequencies of cells that produce IFN-γ (I), TNF-α (T), or MIP-1β (M). The frequencies of cells in unstimulated cultures were subtracted separately for each population. Subpopulations of cells producing different combinations of these three cytokines are displayed with a different color as shown in the graph legends. For example, IFN-secreting cells include IFN-γ+, IFN-γ/TNF-α+, IFN-γ/MIP-1β+, and IFN-γ/TNF-α+MIP-1β+ cells.
Sequential stimulation of dengue-immune PBMC by different variant peptide sequences directly reveals cross-reactive T cells

In the experiments described above, PBMC immune to one serotype responded by producing cytokines when stimulated with peptides from other serotypes, suggesting that these epitopes could stimulate cross-reactive responses. However, the results of these experiments did not indicate whether the same population of epitope-specific cells was responding to the variant peptides or whether each variant peptide activated a different population of epitope-specific T cells. To understand this, we developed an assay which would measure functional responses of the same cell population to two different peptide stimuli.

PBMC were stained with CFSE at the time of stimulation with the homologous peptide (first stimulus). After 5 or 14 days, the cells were washed and restimulated for 6 h with the same or heterologous peptides and stained for cytokine production (second stimulus). The cross-reactive population would be represented by the CFSE-low cytokine+ group, the population specific for the homologous peptide would be represented by the CFSE-low cytokine- subset and the population specific only for the second heterologous peptide would be represented by the CFSE-high cytokine+ subset.

Stimulation of dengue-immune PBMC with the homologous D3 NS4b 2423 peptide induced proliferation of epitope-specific CD8+ T cells. By day 5, fully divided CD3+ CD8+ cells constituted ~16% of the culture and increased to ~48% by day 14 (Fig. 5A). Restimulation with homologous peptide on day 14 induced IFN-γ+ production by 67% of the divided cells. Interestingly, 1.2–1.5% of the cells that remained undivided at day 5 of culture also produced IFN-γ after restimulation. However, by day 14 of culture, essentially none of remaining undivided or partially divided cells were capable of producing IFN-γ+ after restimulation.

In contrast, only 22% of the proliferated cells were IFN-γ+ following D2 peptide stimulation. The CFSE-low IFN-γ+ subset following D1 and D4 peptide stimulation were <5%. This suggests that one-third of the expanded D3 NS4b 2423-specific T cell population is cross-reactive for D2 NS4b 2423 but has very little cross-reactivity for the D1 and D4 variants. This result was unexpected because direct ex vivo stimulation with D1, D2, and D4 peptides induced comparable levels of cytokine production (Fig. 4B). However, it is possible that, under long-term culture conditions, clones that are cross-reactive for D2 and D3 sequences alone have a proliferative advantage. This pattern of cross-reactivity was consistent with that seen in NS4b 2423-specific T cell clones obtained from limiting dilution. Most of the clones derived from D3 peptide-stimulated bulk lines were highly cross-reactive for D2 peptide and less so for D1 and D4 variants in cytokysis assays and intracellular IFN-γ secretion (data not shown).

The pattern of cytokine staining by CFSE+ cells in response to re-stimulation with homologous D3 or heterologous peptides differed. The intensity of IFN-γ staining of CFSE+ cells was lower after stimulation with heterologous D2 peptide than after stimulation with homologous D3 peptide. All of the CFSE+ IFN-γ- cells in cultures stimulated by homologous and heterologous peptides coexpressed TNF-α and MIP-1β (Fig. 5B). In contrast, the majority of the CFSE+ IFN-γ- population did not express TNF-α. But interestingly, 10–15% of the CFSE+ IFN-γ- population expressed MIP-1β following restimulation with D2 and D3 peptides, revealing an unexpected pattern of functional heterogeneity represented by this population of CFSE+ IFN-γ TNF-α− MIP-1β+ cells.

Discussion

The selective expansion of cross-reactive CD8+ T cells present in the Ag-specific memory repertoire during immune response to a heterologous viral infection has been demonstrated in several models of viral infection. The response mediated by this reamplified population is not necessarily protective in nature and, in fact, has been shown in many situations to be immunopathologic (3, 4, 13, 29–31). In these studies, immunopathology was associated with attrition of response or an alteration in the nature of the effector functions of CD8+ T cells caused by qualitative differences in T cell signaling (31–33). Thus, differences in amino acid sequence between an original T cell epitope and its variants impacted the functional outcome of a secondary infection. The four serotypes of
dengue virus do not share complete homology, leading to the possibility that CD8⁺ T cell epitopes not conserved between serotypes will function as altered peptide ligands during secondary infection. We have shown before that variant dengue peptides function as partial agonist ligands by stimulating robust cytolysis but weak proliferation and IFN-γ production (19).

Our results from the present study show additional complexity in the pattern of functional responses to heterologous dengue serotypes. In this study, we identified four new HLA-A*02-restricted dengue epitopes and observed high frequencies of CD8⁺ T cells to all four epitopes in four vaccinated individuals. We were able to observe the heterogeneity of functional response induced by the natural variants of these epitopes at the single-cell level and directly demonstrated serotype cross-reactivity among epitope-specific cells via long-term cloning experiments.

The total numbers of functionally active CD8⁺ T cells specific for all four epitopes in each donor ranged from 0.7 to 4%, and the precursor frequencies of cells specific for each epitope as measured by intracellular IFN-γ secretion ranged from 0.12 to 0.64% of CD8⁺ T cells. This is comparable to frequencies of 0.02 to 2.5% of CD8⁺ cells specific for a HLA-A*011-restricted NS3 epitope during acute phase of illness and is higher than frequencies of 50 IFN-γ⁺ cells/10⁶ PBMC seen within 4 mo of infection in patients in Thailand and Vietnam (13, 34).

Staining for IFN-γ, TNF-α, and MIP-1β simultaneously revealed that very few or none of the cells produced all three cytokines, and that the majority of the responding population was single-positive for each cytokine. This apparent heterogeneity of cytokine response in human PBMC has been reported in other studies (20–24, 35). Some variant sequences induced higher frequencies of TNF-α⁺ cells and MIP-1β⁺ than IFN-γ⁺ cells. One explanation for this finding is that these cells contain preformed TNF-α mRNA (36) and store MIP-1β in cytolytic granules (37), which would lead to faster secretion kinetics for these two cytokines. However, this implies that, at a time point later than that used in the assay, the precursor frequencies of cells secreting all three cytokines would be the same, and this has not been determined in our study.

This pattern of heterogeneous cytokine expression could be significant in determining the clinical outcome of the immune response. In addition to having antiviral properties, TNF-α and MIP-1β also are inflammatory cytokines. MIP-1β is a chemoattractant for both monocytes/macrophages and CD8⁺ T cells. Although the recruitment of these cells is important for viral clearance, it may paradoxically result in immunopathogenesis due to higher viral loads following Ab-enhanced infection of monocytes by virus from lysed cells and higher levels of inflammatory cytokines secreted by activated T cells during secondary infection.

The detection of higher numbers of cells producing TNF-α and MIP-1β than IFN-γ after peptide stimulation suggests that these cytokines might be a better indicator of the size of the epitope-specific population. It also would be interesting to analyze the cytokine secretion patterns of these epitope-specific T cells in the context of tetramer staining. This also could help answer the question of which cytokine accurately reflects the actual numbers of epitope-specific cells.

Previous studies from our lab showed that dengue-specific CD4⁺ T cells from immunized subjects showed the highest IFN-γ response to the homologous serotype of whole dengue Ag prepared from Vero cell lysates (38). The CD8⁺ T cell response to individual epitopes did not follow the same trend. A single variant of two of the four epitopes studied induced the highest total cytokine response in all four donors. Also, the D3 sequence of the NS4b 2353 peptide induced cytokine responses in >1.5% of CD8⁺ T cells in three of the four donors. Although this broad immunogenicity of D2 NS4a 2148, D3 NS4b 2423, and D3 NS4b 2353 did not correlate with the algorithm-based predicted binding scores for these variants, it is possible that these three sequences have the highest affinity for HLA-A*0201.

Some epidemiological studies have associated Southeast Asian strains of dengue 2 with high virulence and dengue 3 infections with greater disease severity, compared with other serotypes (39, 40). When the total cytokine responses to all four epitopes of each serotype were compared (Fig. 4e), peptides of the D2 and D3 serotypes induced the highest responses in all donors. Although responses to a broader group of epitopes would need to be examined in more subjects, these preliminary findings are consistent with the hypothesis that secondary infection with these two serotypes might result in enhanced cytokine response and result in immunopathology.

For a dengue vaccine to work optimally in endemic areas with multiple cocirculating serotypes, the immune responses targeted against T cell epitopes should be broadly cross-reactive. It also should activate a repertoire of high-avidity T cell clones that would effectively clear virus-infected cells via cytolysis, increased production of antiviral cytokines but decreased levels of proinflammatory cytokines. We have identified a novel panel of HLA-A*02-restricted T cell epitopes that stimulate high frequencies of CD8⁺ T cells, induce broad cross-reactive responses, and alter the pattern of cytokine production and proliferation in vaccine-induced memory cells. These changes are unique for each epitope sequence and are influenced by the vaccination serotype and also by the serotype of the secondary in vitro infection. All these factors should be taken into account while designing vaccines that will enhance protective immunity while avoiding immunopathology.

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Disclosures

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