Cross-Reactive Memory CD8⁺ T Cells Alter the Immune Response to Heterologous Secondary Dengue Virus Infections in Mice in a Sequence-Specific Manner

Coreen M. Beaumier, Anuja Mathew, Hema S. Bashyam,a and Alan L. Rothman

Center for Infectious Disease and Vaccine Research, University of Massachusetts Medical School, Worcester

Dengue virus is the causative agent of dengue fever and the more-severe dengue hemorrhagic fever (DHF). Human studies suggest that the increased risk of DHF during secondary infection is due to immunopathology partially mediated by cross-reactive memory T cells from the primary infection. To model T cell responses to sequential infections, we immunized mice with different sequences of dengue virus serotypes and measured the frequency of peptide-specific T cells after infection. The acute response after heterologous secondary infections was enhanced compared with the acute or memory response after primary infection. Also, the hierarchy of epitope-specific responses was influenced by the specific sequence of infection. Adoptive-transfer experiments showed that memory T cells responded preferentially to the secondary infection. These findings demonstrate that cross-reactive T cells from a primary infection alter the immune response during a heterologous secondary infection.

Dengue virus (DV) is a member of the Flaviviridae family that infects 50–100 million people worldwide each year and is transmitted by mosquitoes of the species Aedes aegypti. Most infections are asymptomatic; however, symptomatic infections can result in dengue fever (DF) or the more severe dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). There are 4 genetically and immunologically distinct DV serotypes (D1V, D2V, D3V, and D4V). Natural infections with one serotype of DV result in lifelong immunity to that serotype but not to other (heterologous) serotypes. Interestingly, epidemiological data suggest a greater risk of DHF/DSS during secondary infections, and immunopathological mechanisms have been proposed to contribute to DHF risk [1–6]. One suggested mechanism is antibody-dependent enhancement (ADE). ADE occurs when nonneutralizing antibodies resulting from the primary infection increase the uptake of the second infecting DV [7–9]. Another mechanism is cytokine-mediated immunopathology, where cross-reactive memory T cells from a primary infection recognize antigen from the secondary infection, resulting in elevated T cell activation and cytokine production [10, 11].

Human studies have shown the existence of cross-reactive memory T cells and also increased T cell activation and cytokine production in patients with acute dengue disease [10–12]. Mongkolsapaya et al. [13] demonstrated that a large proportion of the activated T cell population during a secondary infection had a higher affinity for antigen from a heterologous serotype, and they postulated that these T cell responses were generated against the serotype from the primary infection, which is consistent with the existence of cross-reactive memory T cells. There are limitations intrinsic to these human studies. In most patients with secondary DV infections, the serotype of
the previous infection is unknown. Because cross-reactive immune responses from sequential flavivirus infections are possible [14, 15], an unknown infection history could complicate interpretation of the immune response to the current DV infection. Prior exposure to other flavivirus infections is also not usually known. As a result, the influence of a specific serotype as primary or secondary infection on the immune response cannot be determined. Also, different HLA genotypes influence the susceptibility of the patients to disease [16–19], and these differences may complicate the analysis of data compiled from patients with different HLA types.

In light of these limitations, an animal model would be useful to examine the immune response to secondary DV infections. Several mouse models have been used to study DV infection. In these cases, either immunodeficient mice were used or immunocompetent mice were infected with a high DV inoculum or were infected intracranially [20–24]. In contrast, immunocompetent mice infected with lower doses of DV by the intraperitoneal (ip) route remain disease free but develop antibody and T cell responses that parallel human immune responses to primary infection. However, the response to sequential DV infections has not previously been described. Given that we previously identified DV T cell epitopes recognized in BALB/c mice, we infected these immunocompetent mice sequentially with low doses of heterologous serotypes of DV. We found enhanced CD8+ T cell responses after secondary infections compared with acute as well as memory phases after primary infection. The increase in T cell response was dependent on the sequence of the infecting serotype and was the result of cross-reactive memory T cells rather than antibodies.

**MATERIALS AND METHODS**

**Viruses.** D1V strain Hawaii, D2V strain New Guinea C (NGC), D3V strain CH53489, and D4V strain 814669 were used in our experiments. All viruses were propagated in C6/36 mosquito cells, and titers were determined by plaque assay in Vero cells.

**Peptides.** Kd-restricted NS3 and Ld-restricted E epitopes of D2V were characterized by our laboratory [25]. Peptides with amino acid sequences corresponding to each epitope for each serotype were synthesized at the University of Massachusetts Peptide Core Facility (table 1). The amino acid sequences for the NS3 epitope in D1V and D3V are identical, as are those in D2V and D4V, and the resulting peptides are referred to as D1/3 NS3 and D2/4 NS3. The peptides corresponding to the E epitope for each serotype are referred to as D1E, D2E, D3E, and D4E.

### Table 1. T cell epitopes used in this study.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>MHC</th>
<th>Serotype</th>
<th>Sequencea</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS3 (293–306)b</td>
<td>Kd</td>
<td>D1/3</td>
<td>GYSTRWM</td>
</tr>
<tr>
<td>E (331–339)c</td>
<td>Ld</td>
<td>D1</td>
<td>APCKPF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D2</td>
<td>SPCKPE</td>
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<td></td>
<td></td>
<td>D3</td>
<td>APCKV</td>
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<tr>
<td></td>
<td></td>
<td>D4</td>
<td>APCKV</td>
</tr>
</tbody>
</table>

**NOTE.** MHC, major histocompatibility complex.

a Anchor residues are shown in boldface; differences in amino acid sequences between serotypes for each epitope are shown in italics.

b The peptide sequences shown are from D1V strain Hawaii (GenBank accession no. ABG75666), D2V strain New Guinea C (AB202784), D3V strain CH53489 (AAA73199), and D4V strain 814669 (P09866).

c The peptide sequences shown are from D1V strain Hawaii (GenBank accession ABG75666), D2V strain New Guinea C (AAC9275), D3V strain H87 (P27915; fully conserved in strain CH53489), and D4V strain 814669 (P09866).

**Immunization.** BALB/c mice 4–6 weeks of age were purchased from Jackson Laboratories and immunized ip with $2 \times 10^5$ pfu of DV or an equivalent volume of C6/36 culture supernatant. For secondary infection, mice were immunized ip 28–56 days after primary infection with $2 \times 10^5$ pfu of heterologous or homologous DV serotypes. At the indicated time points, mice were killed and underwent splenectomy. Single-cell suspensions were made. Mice were maintained in the Animal Facility at the University of Massachusetts Medical School, in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

**Interferon (IFN)--γ enzyme-linked immunospot (ELISpot) assay.** Peptide-specific IFN-γ-secreting T cells were quantified by ELISpot assay, as described elsewhere [26]. Briefly, wells of 0.45-μm filter plates (Millipore) were precoated with purified anti–mouse IFN-γ monoclonal antibody (M Ab) (AN18; Mabtech) in PBS overnight. Splenocytes ($2.5 \times 10^7$/well) were incubated overnight with 4 μg/mL peptide, 5 μg/mL concanavalin A (Sigma-Aldrich), or medium alone. Wells were washed. Biotinylated anti–mouse IFN-γ MAb (R4–6A2; Mabtech) was added and incubated at room temperature for 2 h. Wells were washed. Streptavidin–horseradish peroxidase (Mabtech) was added and incubated at room temperature for 1 h. Wells were washed. Nova Red substrate solution (Vector Laboratories) was added and incubated for 15 min at room temperature. Plates were then washed with tap water and allowed to dry. Spots were counted either manually or by use of an ImmunoSpot ELISpot plate reader (CTL). All data shown are values for which the medium-only (control) backgrounds were subtracted. The median background value was 7 sfc/1 $\times 10^6$ splenocytes.
**Intracellular cytokine staining (ICS).** Peptide-specific IFN-γ and tumor necrosis factor (TNF)-α–secreting T cells were quantified by ICS assay, as described elsewhere [26]. Briefly, \(5 \times 10^5\) splenocytes were incubated with 10 μg/mL peptide, PMA/ionomycin, or medium alone with 5 μL/mL brefeldin A (GolgiPlug; BD Biosciences) for 5 h at 37°C. Cells were washed twice with FACS buffer (2% fetal bovine serum and 0.1% sodium azide in PBS). Anti-CD16/CD32 (2.4G2; BD Biosciences) was added to each sample and incubated at 4°C for 30 min. Cells were washed. Cytofix/Cyanate (5.3-C711; eBioscience) were added, and the samples were incubated with medium) were subtracted from those of peptide-stimulated cells.

**Adoptive transfer.** Thy1.2+ splenocytes (\(2 \times 10^7\)) from D3V-immune or naive BALB/c mice that were immunized at least 28 days previously were transferred in 100 μL intravenously into congenic Thy1.1+ BALB/c mice. The next day, mice were infected with \(2 \times 10^5\) pfu D2V ip. On day 9 after infection, mice were killed and underwent splenectomy.

**Thy1.2+ cell depletion.** Cells were incubated with Thy1.2 microbeads (Miltenyi Biotec) at 4°C for 15 min. Cells were washed. The cell suspension was applied to an LD depletion column (Miltenyi Biotec) in a MidiMACS separator (Miltenyi Biotec).

**Serum transfer.** On day −1, naive male BALB/c mice were passively immunized ip with 200 μL of undiluted serum from D3V- or control-immunized mice. On day 0, mice were immunized ip with \(2 \times 10^5\) pfu of D2V NGC. On day 9 after infection, mice were killed.

**Statistics.** Medians, confidence intervals, and standard errors were computed by use of SPSS and Microsoft Excel. ELISpot and ICS data were compared between groups by use of the Mann-Whitney U test. \(P < .05\) was considered to indicate significance; all \(P\) values <.10 are shown to illustrate non--statistically significant trends.

## RESULTS

### Kinetics of IFN-γ responses to primary and secondary DV infections in mice.

IFS-γ ELISpot assays were used to quantify the T cell response in DV-infected mice. Mice were immunized with a primary DV infection. On days 4–14 after infection,
the splenocytes were assayed for IFN-γ production in response to the previously identified NS3 and E epitopes corresponding to each serotype (table 1) [25]. The peak response was seen between days 8 and 10 (table 2). The highest overall response was seen after primary D4V infection. D2V gave the next highest response, whereas D1V and D3V gave low responses. The NS3 epitope was immunodominant compared with the E epitope in all immunization groups. In addition, the D2/4 NS3 peptide elic-
ited a larger response than did the D1/3 NS3 peptide, regardless of the infecting serotype.

Twenty-eight days after primary infection, mice were boosted with a secondary infection of a heterologous DV serotype. On days 4–14 after secondary infection, cytokine production of the splenocytes was measured. Although the magnitude of the response was greater after secondary infection than after primary infection, the timing of the peak response was similar, at 8–10 days after infection (table 2). The NS3 epitope was immuno-dominant compared with the E epitope after secondary infection, and the D2/4 NS3 peptide elicited a higher response than did the D1/3 NS3 peptide. Because IFN-γ responses to a non-structural protein were boosted by secondary infection, these data suggest that sterilizing immunity did not occur.

Magnitude and specificity of the T cell response to secondary DV infections and sequence dependency. The model of original antigenic sin and data in humans suggest that prior infection with DV generates memory cells that are cross-

Figure 2. Interferon (IFN)-γ enzyme-linked immunospot (ELISpot) responses to the dengue virus (DV) E epitope. Mice were administered sequential heterologous and homologous DV infections, as described in Materials and Methods. IFN-γ responses to the D1E (leftmost column), D2E (second column), D3E (third column), and D4E (rightmost column) peptides were measured 8–10 days after infection by ELISpot assay. Data are represented as described in the figure 1 legend.
95% confidence intervals are shown. Overall, heterologous secondary infection showed an increase in the frequencies of epitope-specific IFN-γ-secreting cells compared with the response during primary infection with the challenge serotype (figure 1). However, the effect was sequence dependent. A boost in the immune response to D1/3 NS3 and D3E peptides was seen in the group of mice that received a primary infection with D3V followed by a secondary infection with D2V. These observations were not universal in all sequences, and the epitope to which the enhanced response was directed corresponded to the primary infecting serotype.

**Preferential activation of memory T cells during heterologous secondary DV infections.** Because ADE is a proposed mechanism of dengue immunopathology, we considered the possibility that preexisting antibodies from the primary infection could enhance the secondary infection, resulting in the boost in T cell response observed. Therefore, we tested whether passive immunization of mice with DV immune serum would enhance the response to immunization with a heterologous serotype. Mice were given serum from mice immunized with D3V 28 days previously and were challenged with D2V. The IFN-γ ELISpot response was measured on day 8. The T cell response from mice that received the immune serum was lower than that from the mice that received the control serum (figure 3). The decrease in response after adding the serum could be the result of inhibition of infection by neutralizing antibodies in the serum. These data showed that the boosted T cell response we observed after heterologous secondary infection was not due to enhanced antigen presentation by antibody-complexed virus.

Figure 3. T cell responses to infection after passive immunization of mice with dengue virus (DV) immune serum. Male BALB/c mice (4–6 weeks old) were given 200 μL of D3V-immune or naive serum intraperitoneally (ip). The following day, mice were infected ip with 2 × 10^5 pfu D2V. On day 8 after infection, the IFN-γ response was measured by enzyme-linked immunospot assay. Median values (n = 4 per group) and 95% confidence intervals are shown.

These memory cells have a lower threshold of activation than naive cells that are specific to the secondary infecting DV and therefore dominate the immune response to the secondary infecting serotype of DV. To determine whether sequential infections have similar effects on the immune response in this model, mice were sequentially infected with all combinations of 2 DV serotypes. On days 8–10 after primary infection, the splenocytes from subgroups of mice were analyzed in an IFN-γ ELISpot assay. The remaining mice were given a secondary immunization. On days 8–10 after secondary infection, ELISpot assays were performed.

Overall, heterologous secondary infection showed an increase in the frequencies of epitope-specific IFN-γ-secreting cells compared with the response during primary infection with the challenge serotype (figure 1). However, the effect was sequence dependent. A boost in the immune response to D1/3 NS3 and D3E peptides was seen in the group of mice that received a primary infection with D3V followed by a secondary infection with D2V. These observations were not universal in all sequences, and the epitope to which the boosted immune response was directed varied (figure 1 and 2).

A change in the epitope hierarchy was observed after heterologous secondary DV infections. The most striking example was the immune response to the D1/3 NS3 epitope. When mice were challenged with D2V after a primary D3V infection, the immune response to the D1/3 NS3 epitope was significantly increased compared with the response during the memory phase after a primary D3V immunization (figure 1). Another such scenario occurred after a primary D2V infection followed by a D1V challenge, where the immune response to the D2E epitope was boosted over that after a primary D1V infection (figure 2). In both of these cases, the epitope to which the enhanced response was directed corresponded to the primary infecting serotype.

Preferential activation of memory T cells during heterologous secondary DV infections. Because ADE is a proposed mechanism of dengue immunopathology, we considered the possibility that preexisting antibodies from the primary infection could enhance the secondary infection, resulting in the boost in T cell response observed. Therefore, we tested whether passive immunization of mice with DV immune serum would enhance the response to immunization with a heterologous serotype. Mice were given serum from mice immunized with D3V 28 days previously and were challenged with D2V. The IFN-γ ELISpot response was measured on day 8. The T cell response from mice that received the immune serum was lower than that from the mice that received the control serum (figure 3). The decrease in response after adding the serum could be the result of inhibition of infection by neutralizing antibodies in the serum. These data showed that the boosted T cell response we observed after heterologous secondary infection was not due to enhanced antigen presentation by antibody-complexed virus.

These findings suggested that serotype–cross-reactive epitope-specific memory T cells from the primary infection were being preferentially recalled during the heterologous secondary infection, resulting in the boost of the immune response. To determine if this was the case, D3V-immune or naive Thy1.2+ splenocytes were adoptively transferred intravenously into congenic Thy1.1+ mice and infected with D2V. Nine days later, the IFN-γ response was measured by IFN-γ ELISpot and ICS assay. To distinguish between the donor and recipient cells' response by ELISpot assay, the splenocytes were depleted of the Thy1.2+ donor cells before the assay. The response of the depleted splenocytes was compared with the response of the undepleted splenocytes (figure 4A). Depleting the memory cells significantly decreased the response, showing that the memory cells were responsible for the majority of the immune response during secondary infection.

To directly compare the peptide-specific responses of donor and recipient T cells, ICS assays were done after the adoptive transfer using the Thy1 marker to distinguish the donor and recipient cells. A significantly higher frequency of the donor CD8+ T cells responded to peptide stimulation than the endogenous CD8+ T cells. Figure 4B shows data from 1 animal that received D3V-immune Thy1.2+ splenocytes before D2V challenge; 0.6% and 1.3% of donor CD8+ T cells responded to D1/3 NS3 and D2/4 NS3, respectively, compared with 0.11% and 0.17% of host CD8+ T cells. Three adoptive-transfer experiments (n = 11 mice) yielded comparable results. The median frequencies of host CD8+ T cells responding to D1/3 NS3 and D2/4 NS3 were 0.05% and 0.2%, respectively, and the median frequencies of donor CD8+ T cells responding to D1/3 NS3 and D2/4 NS3 were 0.44% and 0.53%, respectively. The difference in the frequency of CD8+ donor ver-
sus host cells secreting IFN-γ in response to D1/3 NS3 was statistically significant ($P = .016$). These results directly show that cross-reactive memory T cells were preferentially recruited during the secondary DV infection.

**Enhanced TNF-α production after heterologous secondary DV infections.** In human studies, TNF-α has been implicated in dengue immunopathology [26, 28, 29]. Both DV-infected cells and DV-specific T cells have been shown to be capable of TNF-α secretion [26, 30]. We therefore determined, using ICS, whether there was an enhanced TNF-α response in mice after secondary infection compared with that after primary infection (figure 5). Similar to what was seen for the IFN-γ response, a boost in the TNF-α response was observed after heterologous secondary infection. These data indicate cross-reactive TNF-α-producing memory T cells were expanded in secondary infection and suggest that there is an altered cytokine-secretion profile in secondary infections compared with primary infections.

**DISCUSSION**

To our knowledge, this is the first study to analyze immune responses in mice after heterologous sequential DV infections.
Our focus was on several aspects of the T cell immune response: specificity, kinetics, frequency, and cross-reactivity. After both primary and secondary DV infections, peptide-specific IFN-γ responses were detected. The detection of a boosted response to epitopes on nonstructural proteins after secondary infection showed that primary infection did not prevent the secondary infection; therefore, sterilizing immunity did not occur, similar to what occurs in humans. When we examined the kinetics of the IFN-γ response after primary and secondary infections, we found that the timing of the peak responses was similar after both immunizations, between days 8 and 10. Although it was initially expected that the onset and peak of the memory response might occur earlier than that after primary infection, the kinetics of the response may reflect minimal antigen load. Given that DV does not replicate well in immunocompetent mice [31], the timing of the T cell response may be accounted for by equally slow production of antigen in both primary and secondary DV infections and/or impeded viral replication in secondary infection due to preexisting antibodies.

After heterologous secondary DV infections, an increase in the frequency of peptide-specific T cells was observed, compared with that seen during acute and memory phases of primary infection with that serotype. The boost in the immune response after heterologous secondary infection in this mouse model is consistent with findings in humans. In humans, increased T cell activation is also associated with more-severe dengue disease [28, 32]. The increased frequency of IFN-γ–producing cells seen after secondary infection in the mice is consistent with observations of increased cytokine production in patients with dengue disease [26, 28, 32]. Interestingly, the magnitude of the T cell response in secondary infection appeared to depend on the sequence of infections. This influence of sequence has been seen in human studies, in which secondary D2V infections were associated with a higher risk of DHF [4, 6]. In BALB/c mice, secondary D2V infection after primary D3V immunizations resulted in the most pronounced boost in T cell response to the peptides studied. However, because of differences in epitopes recognized by humans and mice, the specific sequence in mice is not necessarily indicative of the risk in humans. It is also interesting that the D2/4 NS3 peptide elicited the largest response compared with the other peptides tested regardless of the infecting serotype and sequence of infection. A similar phenomenon was seen in dengue-immune humans, in whom particular epitope sequence variants induced a larger response in vitro regardless of the vaccination serotype [33]. Also, certain D2V strains have been associated with increased virulence, and D3V strains have been linked to increased disease severity [34, 35]. It is possible that sequential D3V and D2V infections may promote immunopathogenesis by resulting in an enhanced immune response.

The observed increased frequency of IFN-γ–producing T cells in secondary DV infection suggested that cross-reactive memory cells from the primary infection were being preferentially recruited during secondary infection. This is very difficult to demonstrate in a definitive way in humans, because preinfection samples are rarely available for study. Although there are limitations in using this system to model human infections (because no viral replication was detected and the animals did not become ill), we confirmed this hypothesis with adoptive-transfer experiments showing that the memory population was the main cell
population responding to the secondary infection. Our data are in agreement with data from clinical studies, in which T cell responses after secondary infection have been shown to be mostly serotype cross-reactive [13, 26, 30]. Our findings of serotype–cross-reactive immune responses in vivo are also consistent with data from Mongkolsapaya et al. [13], who showed that cells from patients with secondary D2V infection had a higher affinity to HLA–peptide tetramers corresponding to other serotypes. These data, along with ours, provide evidence for preferential recruitment of memory T cells during secondary DV infections and also for original antigenic sin of T cells.

The importance of TNF-α in human DV infections has been described previously [26, 28–30, 33]. The ratio of TNF-α–producing IFN-γ–producing T cells among peripheral blood mononuclear cells from dengue-vaccine recipients was greater after in vitro stimulation with antigen from heterologous dengue serotypes [26], suggesting that cross-reactive memory cells produced increased amounts of TNF-α, resulting in more-severe disease. Our data also show that there was a boost in TNF-α production after secondary infection, compared with that during the acute and memory phases of primary infection. These results correlate with human studies and also suggest that cross-reactivity alters the functional profile of the T cells.

We and others have proposed that the increased risk of DHF/DSS after heterologous secondary dengue infections is due to immunopathological mechanisms. It has been hypothesized that increased production of inflammatory cytokines during the immune response resulting from cross-reactive memory cells causes the increased vascular permeability and plasma leakage characteristic of severe dengue disease [11, 36, 37]. Also, it has been previously demonstrated that altered peptide ligands change the functionality of the T cell populations, which may then have a potential immunopathogenic effect [33, 38–40]. Because the 4 serotypes of DV do not share perfect homology, it is conceivable that secondary infections with DV could lead to the stimulation of cross-reactive memory T cells with naturally occurring altered peptide ligands. This stimulation could then change the functional profile of the cells, leading to immunopathology. Our data show that the T cell profile is altered after secondary infection, compared with that observed during primary infection. Therefore, this murine system may be a useful model for the study of selected immunological aspects of DV infection.

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References


