

Differential *In Vivo* Clearance and Response to Secondary Heterologous Infections by H2^b-Restricted Dengue Virus-Specific CD8⁺ T Cells

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Abstract

Cytotoxic T lymphocytes (CTL) are hypothesized to play a role in clearance during primary dengue virus (DENV) infections, and contribute to immunopathology during secondary heterologous infections in humans. We previously reported skewed T-cell responses to secondary DENV infection in BALB/c (H-2^d) mice, reproducing characteristics of human DENV infection. To set the stage for using widely available transgenic and knockout mice, we extended these studies to identify DENV-specific T-cell responses in C57BL/6 (H-2^b) mice. We identified dominant CD8⁺ T-cell responses to H-2D^b-restricted epitopes on the DENV NS4a (aa 249–265) and NS5 (aa 521–537) proteins. High frequencies of IFN- γ - and TNF- α -producing T cells directed at both epitopes were detected following primary infection with all four DENV serotypes, and were augmented by secondary DENV infections. *In vivo* cytotoxicity assays demonstrated rapid clearance of target cells pulsed with the NS4a peptide; in contrast, NS5 peptide-pulsed target cells were poorly cleared *in vivo*. These data characterize two H-2^b-restricted T-cell epitopes displaying divergent *in vivo* function. These results should facilitate further studies of the *in vivo* effects of DENV-specific T cells, including the use of genetically modified mouse strains.

Introduction

DENGUE VIRUS (DENV) IS A FLAVIVIRUS transmitted by the mosquito vector *Aedes aegypti*. It is the causative agent of dengue fever (1), and the more severe dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), which are characterized by plasma leakage. DENV infects 50–100 million people worldwide every year, with 500,000 cases of DHF. There are four immunologically distinct serotypes of DENV (DENV-1, DENV-2, DENV-3, and DENV-4). Lifelong homotypic immunity occurs after infection with one of the serotypes; however, heterologous secondary infections can still occur with any of the other three (2,3).

DHF/DSS is seen with significantly increased frequency during heterologous secondary DENV infections (4–6). Multiple immunopathological mechanisms have been proposed to explain these findings. Infection of monocytes and other Fc γ R-positive cells can be enhanced in the presence of cross-reactive non-neutralizing antibodies by a process known as antibody-dependent enhancement (ADE) (7–9). Cross-reactive memory T cells from the primary DENV infection are hypothesized to be preferentially activated compared to naïve T cells during secondary infection, with more rapid expansion and activation

leading to enhanced production of proinflammatory cytokines, but inefficient clearance of the currently-infecting virus, resulting in DHF/DSS (10–12). Pre-existing antibodies that mediate antibody-dependent cellular cytotoxicity (ADCC) and other immune effector cells have also been proposed to contribute to dengue immunopathogenesis (13–18).

The difficulties in discriminating between these mechanisms in observational studies of naturally-infected humans have led researchers to mimic features of DENV infection in experimental animal models, with limited success (19–25). We previously reported that cross-reactive memory T cells were preferentially activated during heterologous secondary DENV infections in BALB/c mice, resulting in augmented IFN- γ and TNF- α responses, and that this effect was dependent on the serotype sequence of infection (26,27). Although this immunological model replicated aspects of human DENV-specific immunity, responses in BALB/c mice are skewed toward Th-2 responses. In contrast, C57BL/6 (H-2^b) mice are more skewed to a Th-1-type proinflammatory response (28,29). In addition, many genetically engineered mouse strains are available on the C57BL/6 background, which may be valuable for further elucidating the immune mechanisms occurring during dengue infections.

We performed a comprehensive analysis to identify epitopes that elicited a strong T-cell response in C57BL/6 mice by screening overlapping peptides that spanned all of the DENV proteins. We identified and characterized two H-2^b-restricted DENV epitopes. Yauch *et al.* also recently described one of these epitopes (30). In our studies we observed different T-cell avidity for the two epitopes that resulted in markedly different clearance of antigen-presenting cells. Lastly, we demonstrated serotype-cross-reactive responses, as well as enhanced cytokine responses, to heterologous secondary DENV infections.

Materials and Methods

Viruses

DENV-1 strain Hawaii, DENV-2 strain New Guinea C (31), DENV-3 strain CH53489, and DENV-4 strain 814669 were used. All viruses were propagated in C6/36 mosquito cells, and their titers were determined by plaque assay in Vero cells.

Peptides

Overlapping peptides corresponding to all of the DENV-2 NSC proteins were obtained from the National Institute of Allergy and Infectious Diseases Biodefense and Emerging Infections Research Resources Repository (BEI Resources). Peptides ranged from 14–17 amino acids in length, with 11 amino acid overlaps. Peptide pools for each protein were made with 9–12 overlapping peptides in each pool. Truncated peptides were purchased from Anaspec Inc. (San Jose, CA).

Immunization

C57BL/6 mice (4–6 wk of age) were purchased from Jackson Laboratories (Bar Harbor, ME) and immunized IP with 2×10^5 pfu of DENV, or an equivalent volume of uninfected C6/36 cell culture supernatant. For secondary infections, mice were immunized 28–56 d after the primary infection IP with 2×10^5 pfu of heterologous or homologous DENV serotypes. The time points were determined by previously performed kinetics studies of the post-infection immune response (26). At the indicated time points, the mice were sacrificed and splenectomized and single-cell suspensions were made. To determine the MHC restriction of the epitopes, H2-D^b-knockout and H2-K^b-knockout C57BL/6 mice (Taconic Farms, Hudson, NY) were used. The mice were maintained in the Animal Facility at the University of Massachusetts Medical School following AAALAC guidelines.

IFN- γ ELISPOT assays

Peptide-specific IFN- γ -secreting T cells were quantified by ELISPOT as previously described (32). Spots were counted either manually or with a CTL Immunospot ELISPOT plate reader. All data shown represent values after subtraction of the media control (median 5.5 spot-forming cells [SFC] per million splenocytes).

Intracellular cytokine staining

Peptide-specific IFN- γ - and TNF- α -producing T cells were quantified by intracellular cytokine staining (ICS) assay as

previously described (32). The cells were stimulated for 5 h with the peptides at 10 μ g/mL total peptide concentration. The antibodies to surface molecules used were: anti-mouse CD3 ϵ (145-2C11; BD Biosciences Pharmingen, Franklin Lakes, NJ), anti-mouse CD4 (GK1.5; BD Biosciences Pharmingen), and anti-mouse CD8 α (5.3-6.7; eBioscience, San Diego, CA). Intracellular staining was done using anti-mouse TNF- α (MP6-XT22; BD Biosciences Pharmingen), and IFN- γ (XMG1.2; BD Biosciences Pharmingen). Data were acquired by the Flow Cytometry Core Laboratory at the University of Massachusetts using a FACSCalibur, and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR). A small lymphocyte gate was drawn on forward- and side-scatter low populations, and further gated on either CD3⁺ CD8⁺ cells or CD3⁺ CD4⁺ cells. The cytokine-positive gate was drawn on the CD4⁺ or CD8⁺ and IFN- γ ⁺ or TNF- α ⁺ populations. Background frequencies of IFN- γ ⁺ or TNF- α ⁺ cells (from cells incubated with media) were subtracted from those of peptide-stimulated cells.

In vivo cytotoxicity

Assays were performed following the method of Jellison *et al.* (33). Splenocytes from naïve C57BL/6 mice were incubated with peptide (0.01–10 μ M) for 45 min at 37°C. The cells were washed twice with phosphate-buffered saline (PBS). Carboxyfluorescein succinimidyl ester (CFSE, 5, 1.5, or 0.4 μ M; Molecular Probes, Eugene, OR) was added to the different peptide-pulsed target cells. Dodecyltrimethylammonium chloride (DDAO, 2 μ M; Invitrogen, Carlsbad, CA) was also added to the targets. The cells were incubated for 15 min in a 37°C water bath. The cells were then washed two times with PBS, and 1×10^7 cells per target per mouse were transferred IV into DENV-2-immune mice in a volume of 200 μ L. The mice were sacrificed 16 h later and splenocytes were isolated. The cells were fixed and analyzed by flow cytometry. DDAO⁺ cells were selected and then analyzed for the presence of CFSE⁺ cells. Specific killing was calculated as $(1 - [\text{ratio immune}/\text{ratio naïve}]) \times 100$, with ratio = number of events peptide-coated target/number of events no peptide target ($n = 4$ mice per group).

Statistical analysis

Medians, confidence intervals, and standard errors were computed using SPSS and Microsoft Excel. ELISPOT and ICS data were compared between groups using the Mann-Whitney *U* test. *p* Values <0.05 were considered significant, and all *p* values <0.10 are shown to illustrate non-statistically significant trends.

Results

Definition of H-2^b-restricted dengue viral epitopes

At the time we initiated these studies, bulk culture cytotoxicity assays in DENV-immunized C57BL/6 mice had demonstrated recognition of DENV non-structural proteins, but no epitopes had been identified (34). We immunized C57BL/6 mice with a low dose (2×10^5 pfu) of a laboratory strain of DENV-2, and 7 d later we tested splenocytes from infected mice for responses to overlapping peptide pools (12 peptides per pool) that spanned the entire DENV-2 genome in IFN- γ ELISPOT assays (Fig. 1A and 1B). Two peptide

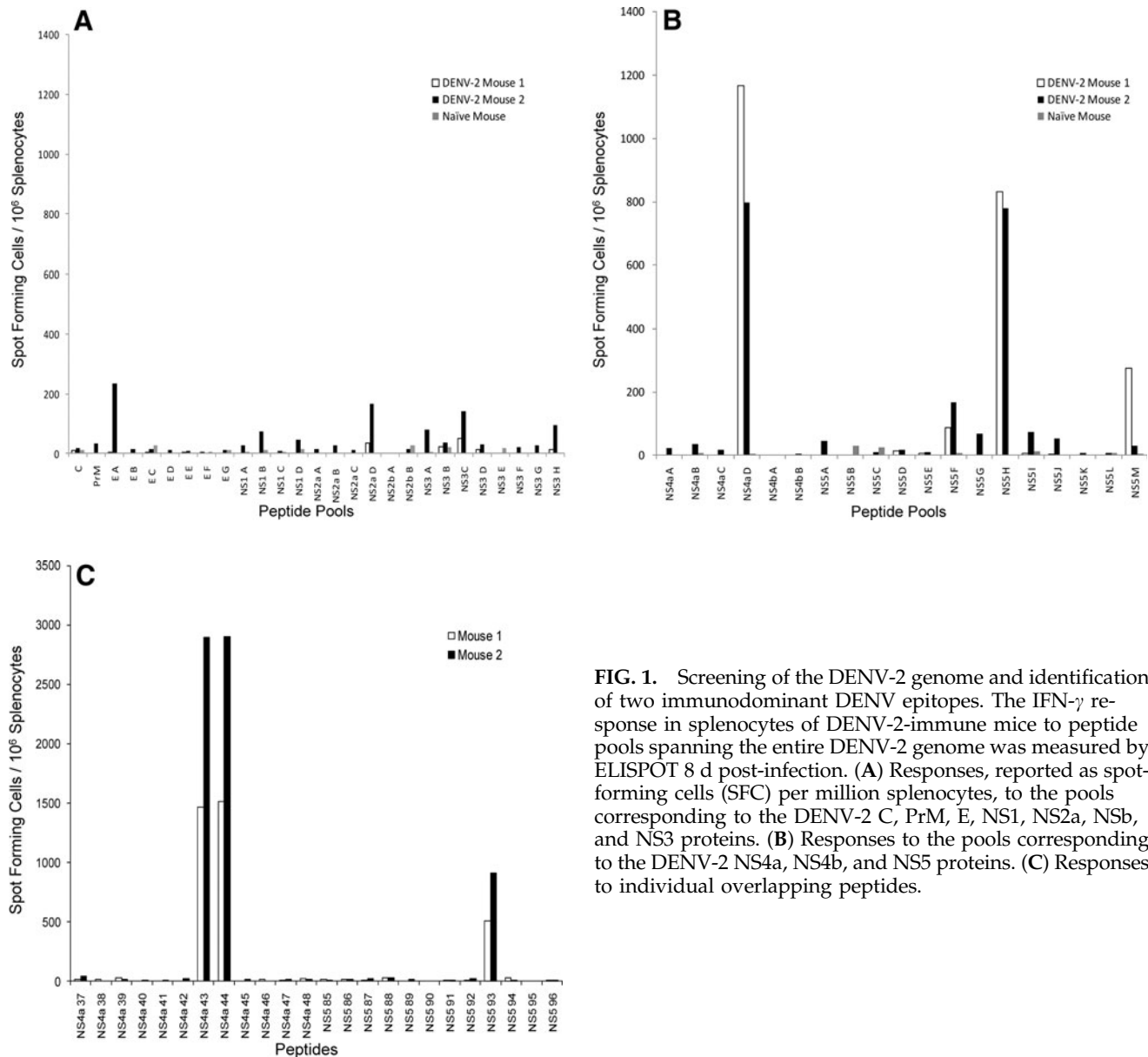


FIG. 1. Screening of the DENV-2 genome and identification of two immunodominant DENV epitopes. The IFN- γ response in splenocytes of DENV-2-immune mice to peptide pools spanning the entire DENV-2 genome was measured by ELISPOT 8 d post-infection. (A) Responses, reported as spot-forming cells (SFC) per million splenocytes, to the pools corresponding to the DENV-2 C, PrM, E, NS1, NS2a, NSb, and NS3 proteins. (B) Responses to the pools corresponding to the DENV-2 NS4a, NS4b, and NS5 proteins. (C) Responses to individual overlapping peptides.

pools, NS4a D and NS5 H, elicited strongly positive IFN- γ responses from DENV-infected, but not naïve, splenocytes (Fig. 1B). Splenocytes from DENV-2-immunized mice were then tested using the individual peptides from positive pools. Two overlapping peptides in the NS4a pool, NS4a 43 (LLAIGCYSQVNPITLTA) and NS4a 44 (YSQVNPITLTAALFLLV), induced IFN- γ production, while a single peptide in NS5 pool H, NS5 93 (DVSKKEGGAMYADDTAG), elicited a positive response (Fig. 1C).

To establish whether these epitopes were recognized by CD4⁺ or CD8⁺ T cells, the IFN- γ response from splenocytes of DENV-2-infected mice was measured by flow cytometry. High frequencies of peptide-specific IFN- γ -producing CD8⁺ T cells were detected, whereas IFN- γ responses among CD4⁺ T cells were not significantly higher than background (Fig. 2A). These data demonstrate that CD8⁺ T cells responded to both the NS4a 43 and NS5 93 peptides (Fig. 2A).

To characterize the magnitude of the IFN- γ responses to both the NS4a 43 and NS5 93 epitopes, the frequency of CD8⁺ IFN- γ -producing cells was determined in DENV-2-immunized mice using intracellular cytokine staining. A median of 1.38% (range 0.38–1.71%) of splenic CD8⁺ T cells responded to the NS4a 43 peptide by secreting IFN- γ , while frequencies of cells that responded to the NS5 93 peptide were slightly lower (median 0.58% of CD8⁺ T cells, range 0.26–1.79%; Fig. 2B).

To determine the MHC restriction of these epitopes, H-2D^b and H-2K^b knockout mice were immunized with DENV-2, and the IFN- γ responses were measured by ELISPOT. No response to either peptide was seen in the D^b knockout mice, while a response equivalent to that seen in wild-type mice was observed in the K^b knockout mice (Fig. 2C). These data indicate that both the NS4a and NS5 epitopes were recognized by CD8⁺ T cells and were restricted by H-2D^b.

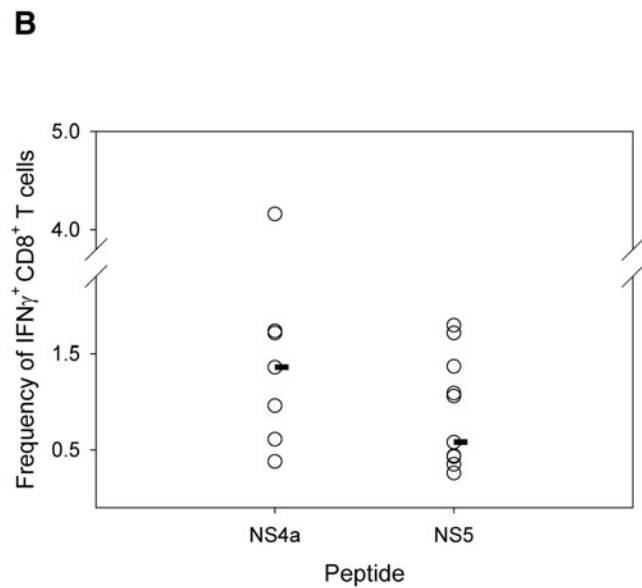
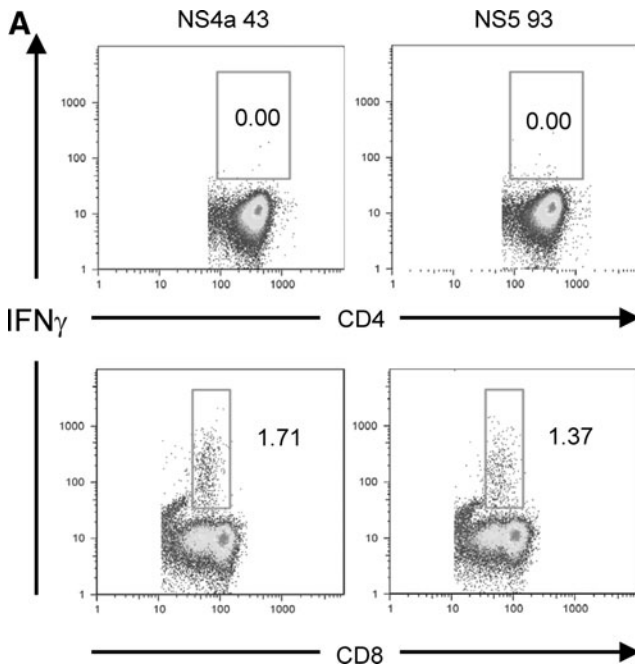
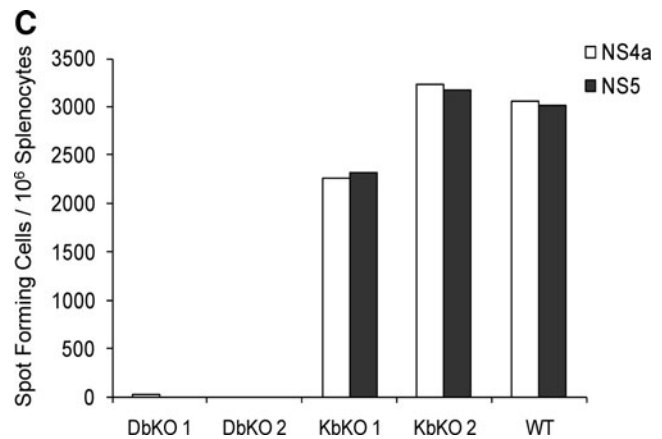


FIG. 2. Characterization of the MHC restriction of the epitopes 8 d post-infection. (A) Representative frequencies of CD4⁺ T cells (top panel) or CD8⁺ T cells (bottom panel) that respond to stimulation with 10 μ g/mL of NS4a and NS5 peptides by intracellular cytokine staining. (B) Median IFN- γ frequencies (n = 7–10 mice) of the NS4a and NS5 epitopes in splenocytes of DENV-2 immune mice by intracellular cytokine staining. (C) ELISPOT responses of splenocytes in H2-D^b and H2-K^b knockout mice immunized with DENV-2. DbKO 1, DbKO 2, KbKO 1, KbKO 2, and WT indicate D^b knockout mouse 1, D^b knockout mouse 2, K^b knockout mouse 1, K^b knockout mouse 2, and wild-type mouse, respectively.



Differential lysis by epitope-specific CTLs

To assess whether epitope-specific CD8⁺ T cells could lyse cells presenting peptide-MHC complexes during the acute response to DENV infection, *in vivo* cytotoxicity assays were performed. Naïve syngeneic splenocytes were pulsed with peptide and adoptively transferred into mice infected with DENV-2 8 d previously. When splenocytes pulsed with 1 μ M of peptide were transferred into acutely infected mice, there was efficient killing of NS4a-pulsed target cells, while lysis of NS5 peptide-pulsed cells was no higher than that of unpulsed cells (Fig. 3A). To determine if the failure to clear NS5 peptide-pulsed cells was due to insufficient expression of peptide-MHC, we repeated these experiments using a range of NS5 peptide concentrations (0.01–10 μ M). We detected minimal killing at NS5 peptide concentrations \leq 1 μ M (Fig. 3B); lysis significantly above the negative control targets was only observed at 10 μ M of NS5 peptide (Fig. 3B). These results demonstrate a difference in *in vivo* cytotoxicity responses to the NS5 and NS4a peptides,

and suggest a difference in avidity of the T cells for these peptides.

Serotype cross-reactive cytokine responses

A key assumption of T cell-mediated immunopathogenesis implies that serotype-cross-reactive memory T cells elicited by primary DENV infection will respond to epitope variants of heterologous DENV serotypes. We identified the optimal NS4a epitope for all four serotypes via IFN- γ ELISPOT after primary DENV-2 infection (Fig. 4A). The amino acid sequences for the minimal NS4a epitopes in D1V and D3V were identical (YSQVNPLTL), and are referred to here as D1/3 NS4a. The DENV-2 epitope was also recently reported on by Yauch *et al.* (30). The peptides corresponding to the NS4a epitope for DENV-2 (YSQVNPITL) and DENV-4 (YSQVNPTTL) are referred to as D2 NS4a and D4 NS4a, respectively. To determine if any cross-reactivity existed in the immune responses to the variant peptides, groups of mice were given a primary infection with each of the four

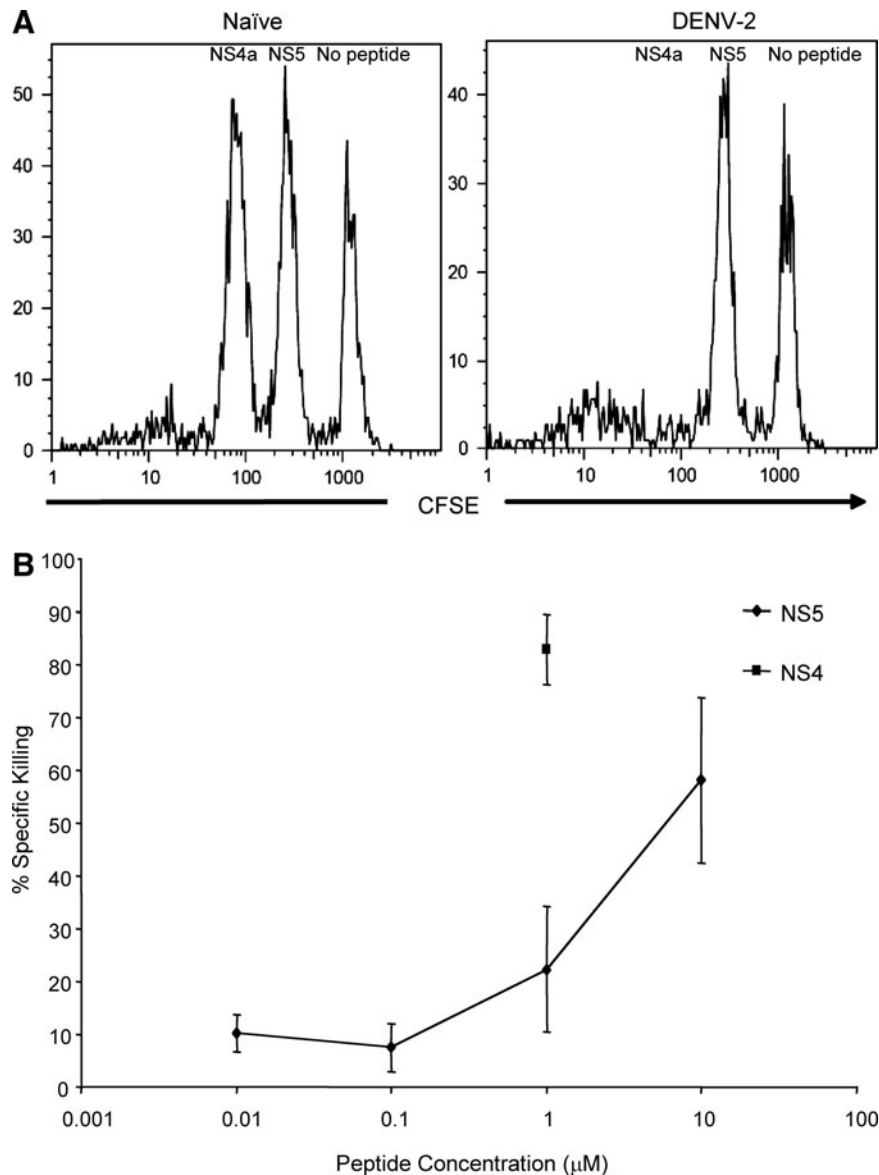


FIG. 3. Differential *in vivo* lysis of NS4a and NS5 peptide-pulsed cells in DENV-2-immune mice. **(A)** Representative examples of loss of CFSE staining in DENV-2-immune mice that received naïve splenocytes pulsed with $1 \mu\text{M}$ of either NS4a or NS5 peptides or no peptide ($n = 4$). Splenocytes were first gated on small lymphocytes and donor cells were further identified by gating on DDAO⁺ cells. **(B)** Summary of specific lytic activity against NS4a- and NS5-pulsed targets in DENV-2-immune mice. Median values are shown for each concentration. Diamonds represent the cytotoxic response to NS5, and squares represent the response to NS4a. Ninety-five percent confidence intervals were calculated.

serotypes, and cytokine responses were measured to the peptides corresponding to all of the different serotypes by intracellular cytokine staining (Fig. 4B and C). Responses were detected after infection with all serotypes. Infection with DENV-2 elicited the greatest response, followed by DENV-3 and DENV-4, while infection with DENV-1 elicited the lowest response (Fig. 4B and C). Immunization of mice with DENV-2, DENV-3, and DENV-4 induced T cells capable of responding to homologous and heterologous NS4a peptides (Fig. 4B), indicating the serotype-cross-reactive nature of the T-cell response.

TNF- α responses were detected in splenocytes after infections with all serotypes, with DENV-2 infections eliciting the largest response, followed by DENV-3, DENV-4,

and DENV-1. Cross-reactive TNF- α ⁺ T-cell responses were also observed. Following DENV-1 infection, higher frequencies of cells responded to heterologous peptides compared to the homologous DENV-1 peptide. Splenocytes from DENV-2-infected mice yielded a low TNF- α response to all of the NS4a peptides. For DENV-3 and DENV-4 infections, some heterologous peptide responses were similar in magnitude to homologous peptide responses. Overall the data indicate that cross-reactive TNF- α responses were detected in mice immunized with each of the four serotypes. These data provide evidence supporting the idea that dengue-specific T cells can secrete TNF- α and IFN- γ in response to stimulation with variants of the NS4a epitope.

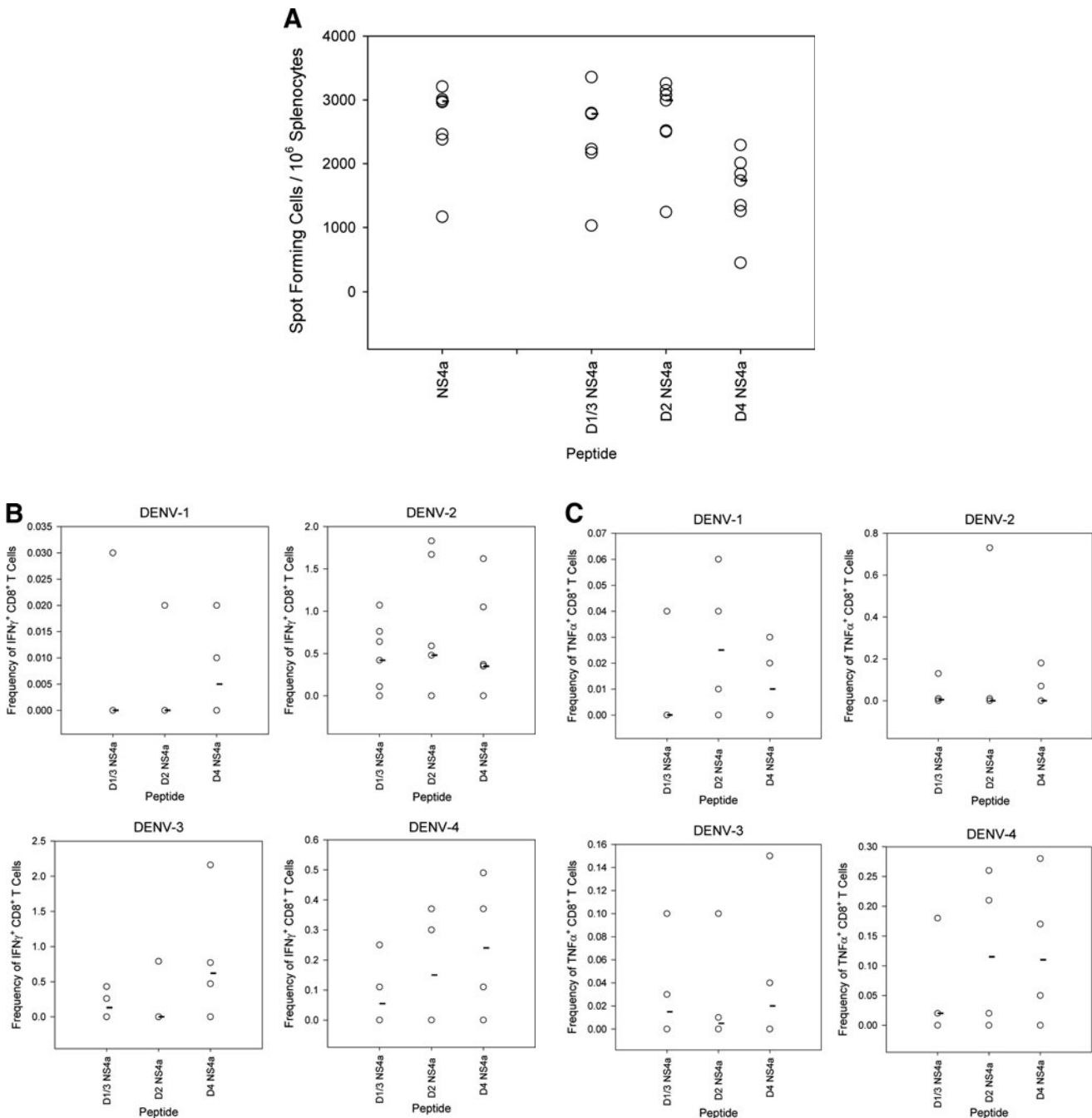


FIG. 4. Serotype cross-reactive cytokine responses to homologous and heterologous variants of the NS4a epitope 8 d post-primary infection. (A) IFN- γ responses to the 15-mer peptide NS4a 43 and to three variants of the 9-mer epitope were measured by ELISPOT assay (B) IFN- γ responses to the variant 9-mer NS4a peptides by intracellular cytokine staining. Values represent frequencies of IFN- γ -secreting CD8⁺ T cells in splenocytes of DENV-immune mice. (C) TNF- α responses to the variant NS4a peptides by intracellular cytokine staining. Values represent frequencies of TNF- α -secreting CD8⁺ T cells in splenocytes of DENV-immune mice. Median values are represented by black bars ($n = 4$ mice per group for all groups, except $n = 7$ for mice receiving primary DENV-2).

Augmentation of immune responses by heterologous secondary DENV infection

We had previously shown that cross-reactive T cells from a primary DENV infection were preferentially recruited to expand during a heterologous secondary DENV infection in BALB/c mice (26). To determine whether memory T cells

from C57BL/6 mice also showed enhanced responses to heterologous secondary DENV infection, we infected mice with DENV-2, and 4 wk later the mice were rechallenged with a secondary DENV-3 infection. T-cell IFN- γ and TNF- α responses to the NS4a variant peptides were measured by intracellular cytokine staining (Fig. 5). Though the IFN- γ response was not significantly augmented (Fig. 5A and C),

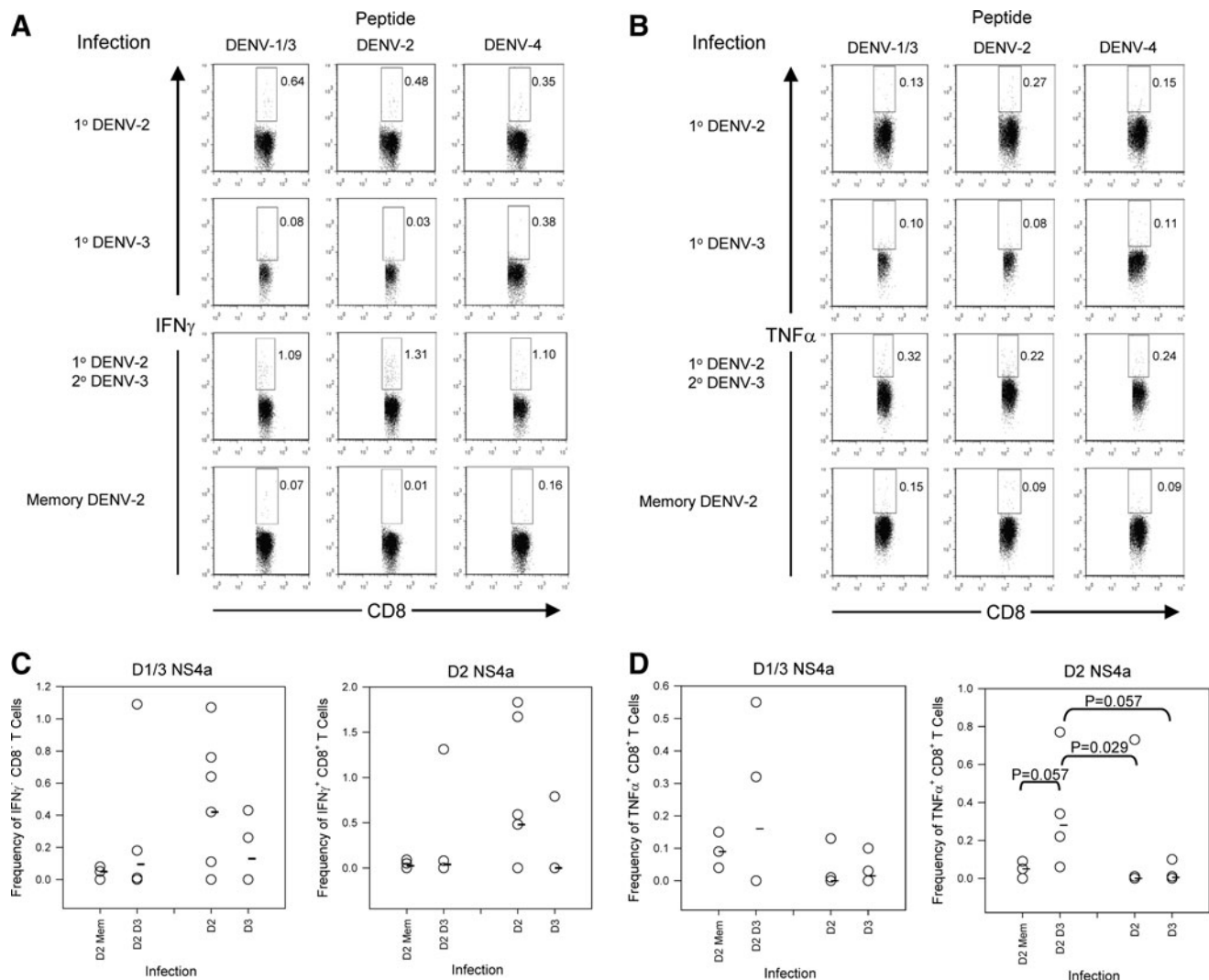


FIG. 5. Cytokine responses following heterologous secondary dengue virus infections. Mice were infected with DENV-2 and challenged 4 wk later with DENV-3. The IFN- γ and TNF- α responses to the variant peptides were measured by intracellular cytokine staining on day 9 after secondary infection. (A and B) Representative cytokine staining for the variant NS4a peptides. 1° DENV-2 is staining of splenocytes 8 d post-primary DENV-2 infection. 1° DENV-3 is staining of splenocytes 8 d post-primary DENV-3 infection. 1° DENV-2 2° DENV-3 is staining of splenocytes 9 d post-secondary infection of DENV-2-immune mice with DENV-3. Memory DENV-2 is staining of splenocytes from mice that received a primary DENV-2 infection 28 d previously. (A) Representative IFN- γ staining for the variant NS4a peptides. Numbers represent the frequencies of IFN- γ ⁺ CD8⁺ T cells. (B) Representative TNF- α staining for the variant NS4a peptides. The numbers represent the frequencies of TNF- α ⁺ CD8⁺ T cells. (C and D) Total IFN- γ and TNF- α responses. The x-axis represents the infection group. Memory phase post-primary DENV-2 infection (D2 Mem), heterologous secondary infection (D2 D3), primary DENV-2 (D2), and primary DENV-3 (D3). (C) IFN- γ response and (D) TNF- α response. Medians are represented by the black bars. The Mann-Whitney U test was used to calculate p values. Only significant values ($p < 0.05$) are shown ($n = 4$ mice per group for all groups, except $n = 7$ for mice that received primary DENV-2).

significant boosting of the TNF- α response was seen to the DENV-2 peptides (Fig. 5B and D).

Discussion

We report the identification of two H-2^b-restricted T-cell epitopes in the DENV NS4a and NS5 proteins, after screening the entire DENV-2 genome in IFN- γ ELISPOT assays. Both epitopes were recognized by CD8⁺ T cells and restricted by H-2D^b. Yauch *et al.* also recently described epitopes in DENV recognized by H-2^b-restricted T cells, including the one we

identified in NS4a, after screening selected candidate MHC-binding peptides (30). Surprisingly, their findings did not include the NS5 epitope that we detected. Significant differences between the design of the two studies may explain the difference in “immunodominant” epitopes identified. We screened the entire DENV polyprotein using overlapping peptide pools, and infected mice with a low dose (2×10^5 pfu) of the prototype DENV-2 strain New Guinea C. In contrast, Yauch *et al.* screened candidate peptides selected using a computer algorithm, not a comprehensive screen of the entire genome, following infection of mice with a much higher dose

(10¹¹ genomic equivalents) of the mouse-adapted DENV-2 strain D2S10. In addition, since they did not report all the peptides that they tested, it was unclear from their study whether or not the NS5 epitope that we describe was tested in their system.

The frequencies of epitope-specific T cells observed in C57BL/6 mice are significantly higher than those we observed earlier in BALB/c mice. This could reflect the greater bias towards Th-1 cytokine production in C57BL/6 mice compared to BALB/c mice (28,29). Alternatively, there may be differences in the expression of DENV antigens *in vivo*; however, this is difficult to assess, as detectable virus is cleared very rapidly in both mouse strains. The higher frequency of DENV-specific T cells in C57BL/6 mice should be an advantage for further studies in this mouse strain.

Using an *in vivo* cytotoxicity assay, we detected killing of target cells pulsed with either the NS4a or NS5 epitope in DENV-2-immune mice, suggesting that these T cells would be active at clearing DENV-infected cells *in vivo*. However, far lower concentrations of peptide were required for killing NS4a peptide-pulsed cells. One possible explanation may be a higher avidity of NS4a-specific T cells for their cognate peptide than the NS5-specific T cells. Yauch *et al.* found that DENV-specific cytotoxic CD8⁺ T cells were protective against lethal D2S10 infection in IFN- α / β R^{-/-} mice (30). NS5 peptide-specific T cells might therefore provide less protective immunity against DENV infection *in vivo*, and instead contribute to immunopathology.

We observed serotype-cross-reactive T-cell cytokine responses to the NS4a peptide in mice immune to any of the four DENV serotypes. Given the cross-reactive nature of the response, we examined the immune response after heterologous secondary DENV infections. Mice infected initially with DENV-2 and subsequently challenged with DENV-3 showed a higher frequency of CD8⁺ T cells producing TNF- α than animals that had had only primary infection with DENV-2 or DENV-3, suggesting that the primary DENV infection altered the secondary immune response. These data are similar to our previous results in BALB/c mice (26,27), as well as findings in natural secondary DENV infections in humans (4–6). Several studies have associated TNF- α levels with dengue disease severity, and T cells have been suggested to contribute to TNF- α production based on *in vitro* studies of antigen-stimulated T cells, as well *ex vivo* staining of PBMCs collected during acute illness (10,32,35–39). The potential for virus-specific T cells to have protective or detrimental effects *in vivo*, as seen in models of heterologous immunity involving unrelated viruses (40–42), needs further investigation, particularly in the context of the development and assessment of DENV vaccines (43). The identification of H-2^P-restricted epitopes should facilitate further studies of the immunological mechanisms of heterologous DENV infection in the mouse model.

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Author Disclosure Statement

No competing financial interests exist.

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