Responses against a Subdominant CD8+ T Cell Epitope Protect against Immunopathology Caused by a Dominant Epitope

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Abstract

CD8+ T cell responses are critical for the control of virus infections. Following infection, epitope-specific responses establish an unpredictable but reproducible pattern of dominance that is dictated by a large number of both positive and negative factors. Immunodomination, or diminution of subdominant epitope-specific responses by dominant epitopes, can play a substantial role in the establishment of epitope hierarchy. To determine the role of a dominant (K^dM2_{82-90}) and a subdominant ($D^bM_{187-195}$) epitope of respiratory syncytial virus in viral control and immunodomination, MHC-binding anchor residues in the two epitopes were mutated individually in recombinant infectious viruses, greatly reducing or deleting the epitope-specific CD8+ T cell responses. Neither mutation negatively affected viral clearance in mice, and compensation by the unmutated epitope was seen in both cases, whereas compensation by five other subdominant epitopes was minimal. Mutation of the dominant K^dM2_{82-90} response resulted in effective viral clearance by the subdominant epitope with less illness, whereas mutation of the subdominant $D^bM_{187-195}$ response resulted in overcompensation of the already dominant K^dM2_{82-90} epitope, and increased severity of illness. Increased illness was associated with poor functionality of the abundant population of CD8+ T cells specific to the dominant K^dM2_{82-90} epitope, as measured by the percentage and magnitude of IFN-γ production. These data demonstrate efficient viral clearance, and a protective effect of subdominant CD8+ T cell responses.

Cytotoxic T lymphocytes are the primary immune mechanism to control infection by viruses and other intracellular pathogens. Viral peptides are processed and presented in the context of MHC class I molecules for recognition by CD8+ T cells (1). The requirements for presentation by MHC class I limit the number of viral epitopes that can be recognized by CD8+ T cells, and responses are often limited to a surprisingly small number of epitopes. Within responding CD8+ T cells, reproducible epitope dominance patterns are often

established, creating an immunodominance hierarchy. Many factors can contribute to epitope hierarchy, including those related to epitope processing and presentation, those inherent to CD8^{+} T cell frequency and function, and regulation imposed by other elements of the immune response or local environment (2–5). The primary factors responsible for the establishment of epitope dominance may vary depending on the system and infection studied, and additional factors may come into play and alter immunodominance during chronic infection or following reinfection (2–9).

Respiratory syncytial virus (RSV) is a virus that can cause severe disease in infants, the institutionalized elderly, and hosts with compromised cellular immunity. It is the major cause of bronchiolitis, pneumonia, mechanical ventilation, respiratory failure, and hospitalization in infants in the United States (10). The role of CD8^{+} T cells in RSV infection is still somewhat controversial. Although some studies show an extensive presence of Ag and a near absence of CD8^{+} T cells in infants that experience severe infection or died of severe RSV (11, 12), we demonstrated that, although not the predominant cell type, CD8^{+} T cells are found in the peribronchiolar and interstitial infiltrates and significantly outnumbered CD4^{+} T cells in the lungs of an RSV-infected infant (13). Robust, systemic virus-specific CD8^{+} T cell responses have been found in infants with severe disease and do not correlate with disease severity (14, 15). In fact, the RSV-specific T cell responses peak during convalescence, and are unlikely to contribute to disease (14). In addition to their role in viral clearance, CD8^{+} T cells have been found to protect against vaccine-enhanced illness after vaccination with formalin-inactived RSV in the mouse model (16).

Immune responses to RSV are often studied in the mouse model, where CD8^{+} T cell responses are strong and sufficient to eliminate the virus, but are also the source of immunopathology and illness (17). We use the CB6F1 mouse model, where both *d*- and *b*-allele responses can be measured simultaneously, and the majority of the response is to two viral epitopes, K $M2_{82-90}$ and D $M_{187-195}$, which respond in a predictable hierarchy (18). To determine the contribution of individual epitope-specific responses, we mutated peptide anchor residues to eliminate or alter the response to the K $M2_{82-90}$ and D $M_{187-195}$ epitopes. Either dramatically reducing an epitope-specific response, or eliminating the response all together resulted in compensation by the other epitope, with little compensation observed by five other subdominant epitopes. This indicates a reciprocity between the $K^d M2_{82-90}$ and the $D^b M_{187-195}$ epitope, and showed that both epitopes immunodominate the other five epitopes during primary RSV infection. Surprisingly, having a quantitatively smaller response to the normally immunodominant $K^d M2_{82-90}$ epitope resulted in reduced illness without reducing the efficiency of viral clearance. Conversely, abrogating the response to the already subdominant $D^b M_{187-195}$ epitope, resulting in overcompensation by the dominant $K^d M2_{82-90}$ epitope, exacerbated illness with no benefit with regard to viral clearance.

Materials and Methods

Generation of live RSV with single amino acid changes in epitope anchor residues

Recombinant epitope-mutated RSV was generated using reverse genetics (19). Point mutations were generated by site-directed mutagenesis of sub-cloned portions of the whole RSV genome using a Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA)

and primers with mutations at the nucleotides of interest. Following confirmation of single amino acid changes, whole genome cDNA constructs with the mutations were generated. All mutations were made in antigenome cDNA 46/6120, with a deletion in the downstream noncoding region of the SH gene and silent modifications in the last few codons of the SH open reading frame previously shown to improve stability of the cDNA without effecting the efficiency of viral replication in vitro or in mice (20). Following antigenome construction, live viruses were rescued as previously described (19, 21). Briefly, BSR T7/5 cells were cotransfected with 5 mg antigenome DNA and plasmids expressing N (2 mg), P (2 μg), M2-1 (1 μg), and L (1 μg) using Lipofectamine 2000. Cells were incubated overnight at 32°C prior to scraping into media and adding to subconfluent monolayers of HEp-2 cells. Cultures were incubated at 32°C, and viruses were harvested 14 d posttransfection. RSV_{wt}, $RSV-M2_{I90T}$, and $RSV-M_{N191S}$ viral stocks for infection were purified by four rounds of plaque purification and derived by sonication of infected HEp-2 monolayers as previously described (22). This method ensures that viral stocks originate from a single viral clone. All epitope mutations were confirmed by RT-PCR of the M and M2 proteins from infected HEp-2 cells following generation of the final stock. HEp-2 cells were maintained in Eagle's MEM containing 10% FBS (10% EMEM), and were supplemented with 2 mM glutamine, 10 U penicillin G per ml, and 10 mg streptomycin sulfate per ml. Cells were determined to be free of mycoplasma contamination by PCR analysis (American Type Culture Collection, Manassas, VA).

Mice and RSV infections

Adult (6- to 10-wk-old) female CB6F1/J mice (The Jackson Laboratory, Bar Harbor, ME) were used for all experiments. All mice were housed in our animal care facility at National Institute of Allergy and Infectious Disease under specific, pathogen-free conditions, and maintained on standard rodent chow and water supplied ad libitum. All studies were reviewed and approved by the National Institutes of Health Animal Care and Use Committee. Mice were anesthetized i.m. with ketamine (40 μg/g body weight) and xylazine (6 μg/g body weight) prior to intranasal inoculation with 3×10^6 PFU live RSV in 100 μl 10% EMEM. Mice were weighed daily postinfection, and percent weight lost was used to assess the severity of illness.

Plaque assays

Mice were sacrificed and lung tissue was removed and quick frozen in 10% EMEM. Thawed tissues were kept chilled while individually ground with mortar and pestle as previously described. Dilutions of clarified supernatant were inoculated on 80% confluent HEp-2 cell monolayers in triplicate and overlaid with 0.75% methyl cellulose in 10% EMEM. After incubation for 4 d at 37°C, the monolayers were fixed with 10% buffered formalin and stained with H&E. Plaques were counted and expressed as $\log_{10}$ PFU/g of tissue. The limit of detection is 1.8 $\log_{10}$ PFU/g of tissue.

Synthetic peptides

RSV $M2_{82-90}$ wild-type (wt) and mutant I90T (SYIGSINNI and SYIGSINNT) and RSV $M_{187-195}$ wt and mutant N191S (NAITNAKII and NAITSAKII) peptides were derived from the RSV M2 and M proteins. Peptides for subdominant epitopes $M2_{127-135}$ (VYNTVISYI),

F_{85-93} (KYKNAVTEL), $F_{250-258}$ (YLTNSELL), $F_{433-442}$ (KTFSNGCDYV), N_{57-64} (ANHKFTGL), and $N_{360-368}$ (NGVINYSVL) were similarly generated from the sequence of RSV-A2. The H2-K^d–binding influenza virus A/Puerto Rico/8/34 $NP_{147-155}$ (TYQRTRALV) peptide, and H2-D^b–binding influenza A/Puerto Rico/8/34 $NP_{366-374}$ (ASNENMETM) peptide were used as negative controls. All peptides were synthesized by Anaspec (San Jose, CA), and confirmed to be >95% pure by analytical HPLC at the National Institute of Allergy and Infectious Disease peptide core facility (Bethesda, MD).

Tetramer and intracellular cytokine staining

Mice were sacrificed and lung and spleen tissues were harvested at various times between days 4–14 postinfection. Spleens were also harvested 60– 100 d postinfection to analyze memory responses. Tissues were disrupted manually between the frosted ends of two sterile glass microscope slides in RPMI 1640 containing 10% FBS (RPMI-10), or by tissue dissociation using a gentleMACS machine (Miltenyi, Germany). Lymphocytes were purified using Ficol LITE at room temperature, washed, then resuspended in RPMI-10. For intracellular cytokine staining (ICS), lymphocytes were incubated at 37°C for 5 h with 1 μM of the appropriate peptide, 1 μg/ml of the costimulatory Abs against CD28 and CD49d, and 1 μg/ml monensin. After the incubation, cells were surface stained with fluorochrome-conjugated Abs against CD3 (145-2C11), CD4 (GK1.5), and CD8 (2.43) then fixed and permeabilized using an ICS kit according to the manufacturer's instructions (BD Pharmingen, San Diego, CA). Intracellular stains were done with labeled Abs to IFN-γ (XMG1.2), IL-2 (JES6-5H4), and TNF-α (MP6-XT22) for 20 min at 4°C. For tetramer analysis, cells were stained with K^dM2_{82-90}, $K^dM2_{127-135}$, or $D^bM_{187-195}$ tetramer (Beckman Coulter, San Diego, CA) during a surface stain also containing Abs for CD3, CD4, and CD8. After staining, cells were washed and analyzed by flow cytometry. Cells were analyzed on an LSR-II (Becton Dickinson, San Jose, CA), and data were analyzed using FlowJo version 8.8.5 (Tree Star, San Carlos, CA). For ICS analysis, Boolean gating was performed after single gating for each cytokine, and background from influenza peptide stimulated control samples was subtracted in Pestle (software provided by Mario Roederer, Bethesda, MD) prior to graphing.

In vivo CTL assays

Lymphocytes were harvested from spleens of naive CB6F1 mice and prepared as targets. After isolation, cells were divided into four samples. One sample of cells was incubated with $M2_{82-90}$ peptide, one was incubated with $M_{187-195}$ peptide, and the remaining two samples were incubated without peptide as controls, all at 37°C for 1 h in RPMI-10. Cells were washed in PBS, and resuspended in 1 mL PBS per sample. The $M2_{82-90}$-pulsed cells were incubated with a high concentration of CFSE (CFDA-SE, 2 mM, Molecular Probes, Eugene, OR), whereas one control sample was stained with a low concentration of CFSE (0.1 μM). $M_{187-195}$-pulsed cells were incubated with high concentrations of chloromethylbenzoylaminotetramethylrhodamine (CMTMR, 25 μM, Molecular Probes), and the final control sample was stained with a low concentration of CMTMR (2.5 μM). All samples were stained for 10 min in a 37°C water bath, then 0.5 ml heat-inactivated FBS was added, followed by a 5-min incubation at room temperature. Finally, the samples were washed three times with RPMI-10, resuspended in PBS, and combined. Mice that had been

infected 7 d prior were injected i.v. in the tail vein with 100 ml of the mixture containing 5×10^6 cells of each of the four labeled populations. Fifteen hours later, lung and spleen lymphocytes were harvested, and the frequency of each of the populations labeled with CFSE or CMTMR was determined by flow cytometry. Cells were injected into naive recipients as controls, and percent specific lysis was determined by comparing the recovered frequency of peptide-pulsed cells to the frequency of cells not pulsed with peptide in infected mice compared with naive controls.

Statistical analysis

Data were analyzed in GraphPad Prism using a one-way or two-way ANOVA, followed by Bonferroni's posttests for multiple comparisons for significance between all groups.

Results

Live RSV tolerates single amino acid changes in anchor residues of the K^dM2_{82-90} and $D^bM_{187-195}$ CD8$^+$ T cell epitopes

To date, several CD8$^+$ T cell epitopes have been mapped in H-2^d (BALB/c) and H-2^b (C57BL/6) strains (23–29). Our laboratory has made use of the H-2$^{d/b}$ CB6F1 hybrid mouse to simultaneously investigate responses to a larger number of epitopes (Table I). The K^dM2_{82-90} is a strongly dominant response in the RSV-infected BALB/c (H-2^d) parental strain with only a small fraction of CD8$^+$ T cells specific for other described epitopes (23–27). In primary infected C57BL/6 mice (H-2^b), the $D^bM_{187-195}$ epitope is similarly dominant, with significantly lower responses to other described epitopes (28, 29). Following infection of the CB6F1 mouse, the K^dM2_{82-90} epitope dominates the CD8$^+$ T cell response and the $D^bM_{187-195}$ epitope becomes subdominant and accounts for 3- to 5-fold fewer tetramer-positive CD8$^+$ T cells than the dominant response. Overall, the combined response to the K^dM2_{82-90} and the $D^bM_{187-195}$ epitopes can account for up to 75% of all CD8$^+$ T cells found in the lungs of infected CB6F1 (18), with other described epitopes (Table I) playing a minor role in the overall CD8$^+$ T cell response.

First, we asked how the response hierarchy would change if a major component of the response was removed. We sought to eliminate the CD8$^+$ T cell response to either the K^dM2_{82-90} or the $D^bM_{187-195}$ epitope of RSV and evaluate subsequent CD8$^+$ T cell responses in infected CB6F1. To abrogate peptide binding to the MHC molecule, the anchor residues of each peptide were targeted for mutation. Site-directed mutagenesis of the RSVA2 antigenome plasmid was performed, and attempts to rescue epitope mutant viruses were performed by transfecting BHK-T7 cells with the antigenome plasmid in conjunction with helper plasmids expressing N, P, M2-1, and L as summarized in the *Materials and Methods* and previously described (19). Successful mutations of the anchor amino acid residues were confirmed by RT-PCR and sequencing of the targeted gene following infection of HEp-2 cells.

Initial attempts were made to change the tyrosine at residue 2 of the $M2_{82-90}$ peptide (SYIGSINNI) to an arginine (Y83R), a mutation previously shown to completely abolish the response to the K^dM2_{82-90} epitope (26). As described by others, live RSV of this genotype

could not be rescued despite numerous attempts, indicating that the mutation most likely negatively affects the function of the M2 protein (30). Likewise, attempts to rescue viruses with threonine (Y83T) or serine (Y83S) at the position 2 anchor were also unsuccessful. Although live RSV could be rescued with a more conservative substitution of another hydrophobic aromatic amino acid at the position 2 MHC binding residue (Y83F), we found that this mutation resulted in a virus that also induced a strongly dominant K^dM2_{82-90} response, indicating that phenylalanine in position 83 may also serve as an MHC-binding anchor residue (data not shown). Finally, live RSV with a mutation of the anchor residue at position 9 from an isoleucine to a threonine (RSV-M2$_{I90T}$) was successfully rescued (Fig. 1A). This mutation is known to result in a 10-fold lower binding affinity of the M2$_{82-90}$ peptide for H2-K^d (31).

The M$_{187-195}$ subdominant response peptide contains MHC-binding anchor residues at amino acid positions 5 and 9. In this case, substitution of the asparagine at position 5 for a serine resulted in a virus that was readily and reproducibly rescued (RSV-M$_{N191S}$, Fig. 1A). Both RSV-M2$_{I90T}$ and RSV-M$_{N191S}$ grew well in cultured HEp-2 cells.

Epitope-mutant RSV replicate like wt in CB6F1 mice and are cleared with similar kinetics

Four rounds of plaque purification were done for each RSV$_{wt}$, RSV-M2$_{I90T}$, and RSV-M$_{N191S}$, and high-titer stocks derived from single isolated plaques were generated as previously described (22). Viral titer typically peaks at day 4 or 5 following intranasal infection of CB6F1 mice with RSV$_{wt}$, and clearance is achieved by 7–8 d postinfection. CB6F1 mice infected with 3×10^6 PFU of wt RSV$_{wt}$, RSV-M2$_{I90T}$, and RSV-M$_{N191S}$ had similar lung viral titer kinetics when measured daily between days 4–9 following infection (Fig. 1B). At day 7, epitope-mutated viruses were cleared better than RSV$_{wt}$ ($p < 0.001$), but each virus was completely cleared by 9 d postinfection.

RSV epitope-specific responses have different abilities to compensate for lost or altered epitopes

RSV epitope-specific CD8$^+$ T cell responses for K^dM2_{82-90}, $D^bM_{187-191}$, as well as the minor $K^dM2_{127-135}$ epitope, were measured in the lungs between 4 and 14 d postprimary infection in mice infected with RSV$_{wt}$, RSV-M2$_{I90T}$, and RSV-M$_{N191S}$. As previously reported (18), RSV$_{wt}$ infection resulted in a strongly K^dM2_{82-90} dominated response, a 4-fold lower response to the $D^bM_{187-195}$ epitope, and near-baseline response to the $K^dM2_{127-135}$ epitope (Fig. 2A). Mice infected with RSV with the dominant epitope mutated, RSV-M2$_{I90T}$, demonstrated a 10-fold reduction in the response to the K^dM2_{82-90} epitope. Of the epitopes tested in this experiment, the $D^bM_{187-195}$ epitope dominated the CD8$^+$ T cell response in RSV-M2$_{I90T}$ mice, with a significantly ($p < 0.05$) higher response to this epitope than in mice infected with RSV$_{wt}$. There was no detectable increase in the $K^dM2_{127-135}$-specific CD8$^+$ T cell in response to the change in K^dM2_{82-90} (Fig. 2A). Finally, mice infected with RSV-M$_{N191S}$ completely lacked a response to the $D^bM_{187-195}$ epitope. Although the minor $K^dM2_{127-135}$ response did not compensate for the loss of the $D^bM_{187-195}$ epitope, the K^dM2_{82-90} response compensated significantly ($p < 0.05$).

To evaluate the extent of compensation by several nearly background CD8$^+$ T cell responses during primary RSV infection, peptides corresponding to five additional epitopes ($F_{85–93}$, $F_{250–258}$, $F_{433–442}$, $N_{57–64}$, and $N_{360–368}$) were pooled and used to stimulate lung CD8$^+$ T cells from infected mice. As measured by cytokine (IFN-γ, TNF-α, and IL-2) production following stimulation, these five epitopes together account for <0.5% of the CD8$^+$ T cell response in the lungs of RSV$_{wt}$ infected CB6F1 mice (Fig. 2B). Following infection with RSV-M2$_{I90T}$ or RSV-M$_{N191S}$, the response to this combination of epitopes peaked at 1.5% of lung-resident CD8$^+$ T cells 11 d postinfection, indicating only slight compensation by one or more peptides within the pool.

Epitope dominance patterns established during primary infection with RSV$_{wt}$, RSV-M2$_{I90T}$, and RSV-M$_{N191S}$ persisted into the memory phase (Fig. 2C). Mice infected with RSV-M2$_{I90T}$ had lower CD8$^+$ T cell memory responses to K^{d}M2$_{82–90}$, and significantly higher ($p < 0.01$) responses to D^{b}M$_{187–195}$ in the spleen than mice infected with RSV$_{wt}$. Conversely, CB6F1 infected with RSV-M$_{N191S}$ had a significantly higher ($p < 0.01$) response to K^{d}M2$_{82–90}$ and no detectable memory response to D^{b}M$_{187–195}$. Responses to K^{d}M2$_{127–135}$ were not significantly different between groups of mice infected with the three different viruses.

Quantitative changes in CD8$^+$ T cell responses following mutant virus infection occur with or without qualitative changes

We compared the CD8$^+$ T cell quantity (as measured by tetramer) to the quality of K^{d}M2$_{82–90}$ and D^{b}M$_{187–195}$ specific responses (capacity to produce cytokines upon stimulation) in the lungs of infected mice. In vitro peptide stimulation can result in down-regulation of the TCR, so parallel samples from the same mouse lungs were stained separately for tetramer or cytokine production after stimulation. As we and others have previously reported (18, 32), the dominant K^{d}M2$_{82–90}$ response has a large disparity between quantity and quality: less than half of the CD8$^+$ T cells recovered from the lungs of mice following RSV$_{wt}$ infection that are specific for this epitope produced cytokine upon stimulation in vitro with the M2$_{82–90}$ peptide (Fig. 3A). Although CD8$^+$ T cells from the lungs of mice infected with RSV-M2$_{I90T}$ contain 10-fold fewer cells specific for the K^{d}M2$_{82–90}$ epitope, these cells were highly functional, and all produced cytokine after stimulation with the M2$_{82–90}$ peptide (Fig. 3A). A side-by-side comparison of K^d M2$_{82–90}$ responses following infection with RSV$_{wt}$ or RSV-M2$_{I90T}$ is presented in Supplemental Fig. 1. The Vβ profile of epitope-specific cells is similar between mice infected with either virus, suggesting that cells responding to the mutant epitope are a subset of those that respond to the wt epitope. Stimulation with either the native M2$_{82–90}$ peptide, or the M2$_{I90T}$ peptide (SYIGSINNT) following infection with either RSV$_{wt}$ or RSV-M2$_{I90T}$ results in expression of IFN-γ by cells with an identical Vβ distribution, indicating that both peptides stimulate the same population of cells (Supplemental Fig. 1). These data are consistent with an alteration in the anchor residue with no changes in the TCR contact residues, and the reported 10-fold lower binding affinity to H2-K^d associated with this mutation (31). Finally, infection with RSV-M$_{N191S}$ resulted in an increased K^{d}M2$_{82–90}$ response, yet as in infection with RSV$_{wt}$, only half of the cells produced cytokine upon stimulation (Fig. 3A), indicating that the compensation was only in magnitude and not quality of the response. Dividing the T

cell quality by the quantity for the K^dM2_{82-90} epitope at all timepoints combined quantifies the proportion of epitope-specific cells capable of producing cytokines. Using this method, it is clear that similar proportions of K^dM2_{82-90} cells produce cytokine in the RSV_{wt} and $RSV-M_{N191S}$–infected groups, and those in $RSV-M2_{I90T}$–infected mice are more highly functional (Fig. 3B). Cells in this final group were also found to produce more cytokine per cell, as reflected by measuring the median fluorescence intensity (MFI) for IFNγ in producing cells ($p < 0.0001$, Fig. 3C). The basis for the improved quality of the response is under investigation.

$D^bM_{187-195}$-specific responses were similarly analyzed in mice infected with RSV_{wt} and $RSV-M2_{I90T}$. Cells specific for this epi-tope were undetectable in mice infected with $RSV-M_{N191S}$, and the mutant peptide (NAITSAKII) did not stimulate any production of IFN-γ following in vitro stimulation of $RSV-M_{N191S}$–infected mice. The altered peptide was, however, able to stimulate the $D^bM_{187-195}$ specific cells following RSV_{wt} infection (data not shown). There were more $CD8^+$ T cells specific for $D^bM_{187-195}$ in mice infected with $RSV-M2_{I90T}$ compared with RSV_{wt}, and most of these cells produced cytokine following stimulation, as observed following infection with RSV_{wt} (Fig. 3D). Although the proportion of IFN-γ–producing cells was slightly reduced at later time points, the proportion of $D^bM_{187-195}$-specific cells from $RSV-M2_{I90T}$–infected mice that expressed cytokine was similar to that of RSV_{wt}, (Fig. 3E). Between the two groups, there was no significant difference in MFI (Fig. 3F). Overall, more $D^bM_{187-195}$-specific cells were double positive for production of both IFN-γ and TNF-α, than were K^dM2_{82-90} cells, which tend to produce IFN-γ only (Fig. 3D versus 3A).

We measured the ability of K^dM2_{82-90} and $D^bM_{187-195}$-specific cells to kill peptide-pulsed autologous primary lymphocytes as targets with an in vivo CTL assay. Peptide pulsed cells were labeled with high concentrations of CFSE ($M2_{82-90}$) and CMTMR ($M_{187-195}$) prior to i.v. injection into mice that had been infected 7 d earlier. Fifteen hours later, cells were harvested from the lungs and spleen and the proportion of cells that were killed was quantified by comparison with coinjected cells labeled with low concentrations of CFSE and CMTMR that were not pulsed with peptide. In both the lung and the spleen of RSV_{wt}-infected mice, more $M2_{82-90}$-pulsed cells were killed than $M_{187-195}$-pulsed, with virtually all of the $M2_{82-90}$-pulsed cells killed by 15 h post-injection (Fig. 4A). This suggests that an excess of K^dM2_{82-90}-specific cells can sufficiently kill all the transferred targets, or that the discordance in tetramer binding and cytokine production may not be associated with a defect in cytolytic activity. Interestingly, in mice infected with $RSV-M2_{I90T}$, more $M2_{82-90}$-pulsed targets were killed than $M_{187-195}$ pulsed in the lungs ($p < 0.001$); however, nearly equal proportions of both targets were killed in the spleen. These data confirm high functionality of the remaining K^dM2_{82-90}-specific cells in the lungs of mice infected with $RSV-M2_{I90T}$. Finally, nearly all $M2_{82-90}$-pulsed cells were killed in both the lungs and spleen of $RSV-M_{N191S}$–infected mice, whereas all cells pulsed with $M_{187-195}$ remained (Fig. 4A), consistent with the near-absence of $D^bM_{187-195}$-specific $CD8^+$ T cells (Fig. 2A).

Increased illness/immunopathology results from overcompensation by a dominant RSV epitope

Immunopathology in the mouse model of primary RSV infection can be measured by weight loss. Illness caused by the CD8$^+$ T cell response peaks at 7–8 d postinfection. Mice infected with RSV$_{wt}$ experienced a loss of 6.6% of their body weight by day 8, after which they recovered (Fig. 4B). Mice infected with RSV-M$_{N191S}$ experienced more dramatic weight loss earlier than those infected with RSV$_{wt}$, losing almost double (11.3%) the weight lost by mice infected with RSV$_{wt}$ by 7 d postinfection ($p < 0.05$). Although not statistically significant, RSV-M2$_{I90T}$ infected mice tended to lose less weight than mice infected with RSV$_{wt}$, and began their recovery 1 d sooner. This suggests that the subdominant T cell responses make a significant contribution to viral clearance without exacerbating illness.

Discussion

Various studies reporting mutations in epitope-specific CD8$^+$ T cell responses within a virus have reported either little to no compensation by other CD8$^+$ responses (30, 33–35), or more significant compensation by one or multiple epitopes (36, 37). Some report little compensation during primary infection, but a greater amount during secondary infection (33, 34). The ability of epitopes to compensate for the loss of others has been shown to have an effect on the ability to control viral pathogens (30, 37). We demonstrate in this study that changes of even a single amino acid can abolish specific CD8$^+$ T cell responses to RSV during primary infection of CB6F1 mice, alter immunodominance hierarchy and illness, and change the functional properties of the responding T cell population.

In these studies, we saw compensation by other epitopes following mutation of either the dominant K^{d}M2$_{82-90}$ or the sub-dominant D^{b}M$_{187-195}$ response. In the case of mutation of the K^{d}M2$_{82-90}$ (RSV-M2$_{I90T}$), which resulted in one log$_{10}$ reduction in this response, the next dominant D^{b}M$_{187-195}$ epitope provided the most compensation, with only modest compensation by a pool of subdominant responses. Following mutation of the subdominant D^{b}M$_{187-195}$ response (RSV-M$_{N191S}$), which essentially ablated that response, we again found modest compensation by the sub-dominant pool, and "overcompensation" by the already dominant K^{d}M2$_{82-90}$ response. These data clearly show that the subdominant D^{b}M$_{187-195}$ response exerts some level of control over the dominant K^{d}M2$_{82-90}$ response, which is of benefit to the host. This may account for the observation that CB6F1 hybrid mice, which recognize a greater number of subdominant viral determinants, experience more mild disease following infection than the BALB/c parent strain, which has an almost exclusively K^{d}M2$_{82-90}$-specific CD8 T cell response (18).

In contrast to work by Vallbracht et al. (30), we saw no compensation by the subdominant K^{d}M2$_{127-135}$ epitope postinfection with either RSV-M2$_{I90T}$ or RSV-M$_{N191S}$. They mutated the K^{d}M2$_{82-90}$ epitope at position 8 (SYIGSINNI → SYIGSINAI), a mutation in the TCR binding region predicted to affect TCR recognition rather than MHC binding, and saw a complete abolition of the response. Following infection with this virus, they found that the K^{d}F$_{85-93}$ epitope did not compensate at all, but saw a small increase in the response to the K^{d}M2$_{127-135}$ epitope and no measurable responses to previously undetected epitopes. Similarly, Mok et al. (31) saw compensation by the K^{d}M2$_{127-135}$ epi-tope in the context of

multiple rounds of DNA immunization with a plasmid expressing the M2 protein with a mutation in both anchor residues of the K^dM2_{82-90} epitope that abolished the response entirely prior to infection with RSV. Both of these studies were done in BALB/c mice, where the $CD8^+$ response was limited to responses restricted to *d*-allele MHC proteins. This demonstrates that the factors defining the ability of a particular epitope to respond and compensate may differ by mouse strain, and the presence or absence of other MHC molecules. It is possible in our study, that the combined responses to the $D^bM_{187-195}$ and mutated K^dM2_{82-90} following RSV-$M2_{I90T}$ infection, and the overly dominant responses to the K^dM2_{82-90} during RSV-M_{N191S} infection sufficiently immunodominate the $K^dM2_{127-135}$ epitope during infection with either of these viruses and that the response to this epitope is dispensable.

Although one postulated mechanism for immunodomination is the negative effect of production of IFN-γ by dominant epitopes on the subdominant (38), several studies have proven that the outcome of infection after epitope mutation is not easy to predict, and compensation does not always occur by the "next best" epitope (33–35). Responses to some epitopes appear to be more inherently self-limited than others and can only be expanded to a certain extent, whereas minor epitopes previously below the level of detection, or "weak" subdominant responses may arise in some cases (39, 40). Although efforts that extended beyond measuring already defined epitopes within RSV were not taken, we believe that there is a low likelihood for the emergence of a new strong response to an undescribed epitope based on both the lack of illness in the RSV-$M2_{I90T}$–infected group of mice and the strong reciprocal compensation of the two major $CD8^+$ T cell responses.

The mechanism for the enhanced function of the remaining K^dM2_{82-90} response in mice infected with RSV-$M2_{I90T}$ is unknown. Despite a 10-fold reduction in numbers, these cells are functionally superior to K^dM2_{82-90}-specific cells in an infection with RSV_{wt} in both their frequency of cytokine production and the amount of cytokine they produce (Fig. 3B, 3C). We can correlate this increase in function with a strong ability to kill cells that present the $M2_{82-90}$ peptide in vivo in these studies, as $M2_{82-90}$-pulsed cells were as readily, or more readily killed in the spleen and lungs of RSV-$M2_{I90T}$–infected mice than cells pulsed with the $M_{187-195}$ peptide (Fig. 4A). Clonotyping and other analysis of this response is a subject of ongoing investigation.

Several studies suggest that responses to subdominant epitopes are as effective, or more effective, than responses targeting immunodominant epitopes. As one example, dominant responses against the hepatitis B surface Ag severely limit the diversity of the antiviral response. Upon elimination of immunodominant epitopes, subdominant responses were found to have striking antiviral potential (39). In the influenza mouse model system, immunodominant epitopes like the $D^bPA_{224-233}$ have been found to have poor protective efficacy due in part to aspects of Ag presentation following infection (41). Strong responses to this epitope can actually delay viral clearance. Other epitopes of influenza have demonstrated a similar inability to clear virus following epitope-specific immunization (42). It has also been shown that sub-dominant epitopes are responsible for durable control of SIV-mac239 replication in a rhesus macaque model of HIV-1 (43). Responses can be

generated against subdominant epitopes even in the context of ongoing infection and are potentially beneficial in controlling disease (39, 44).

The development of narrowly focused, strongly immunodominant responses, which inhibit the response to subdominant epitopes, may diminish effective control of the pathogen depending on the epitope. Certainly, they are of limited use if the virus adapts to abrogate responses to one or two immunodominant epitopes, as would be likely to occur in more persistent viral infections. We demonstrate in this study that an overall lower CD8$^+$ T cell response, with more codominance between the normally subdominant D^{b}M$_{187-195}$ and the mutant K^{d}M2$_{82-90}$ epitope shows benefit to the host by clearing virus with minimal illness. Following viral infection in vivo, multiple subdominant epitopes working together toward viral clearance that exert pressure on a sufficient number of viral determinants may be most favorable for the host. This strategy to avoid immunodomination would increase immunological flexibility, reduce the risk of escape, and potentially diminish immunopathology.

Designing vaccines that elicit codominant CD8$^+$ T cell responses is a strategy that should be considered for some viral pathogens. Achieving optimal vaccination will require a greater understanding of what constitutes a protective epitope, the role of immunodominance and immunodomination on final outcome, and the basis for CD8 T cell-mediated immunopathology. We have shown in this study that mutation of an immunodominant epitope, which dampens the magnitude of response, diminishes immunodomination, and increases its functionality in vivo, may result in a more efficient CTL response that can clear virus with less immunopathology. Alternatively, eliminating a subdominant epitope may increase dominance of an already dominant epitope, resulting in enhanced CD8$^+$ mediated immunopathology. Defining the properties of epi-topes that produce unfavorable, immunodominating responses that have poor killing efficiency and their counterpart responses that can efficiently clear virus may be necessary to fully exploit T cell-based vaccine approaches. In addition, this study cautions that simple enumeration of vaccine-induced T cell responses may not be a reliable surrogate for vaccine efficacy.

In summary, compensation for mutated CD8$^+$ T cell epitopes is a complex phenomenon. Defining factors that determine the capacity for individual epitopes to compensate, as well as those that confer increases in functionality, will be valuable for the design of immunogens that promote effective responses without the potential for deleterious effects.

Acknowledgments

Abbreviations

|	
CMTMR	chloromethylbenzoylaminotetramethylrhodamine
EMEM	Eagle's MEM
ICS	intracellular cytokine staining

MFI   median fluorescence intensity

RSV   respiratory syncytial virus

wt   wild-type

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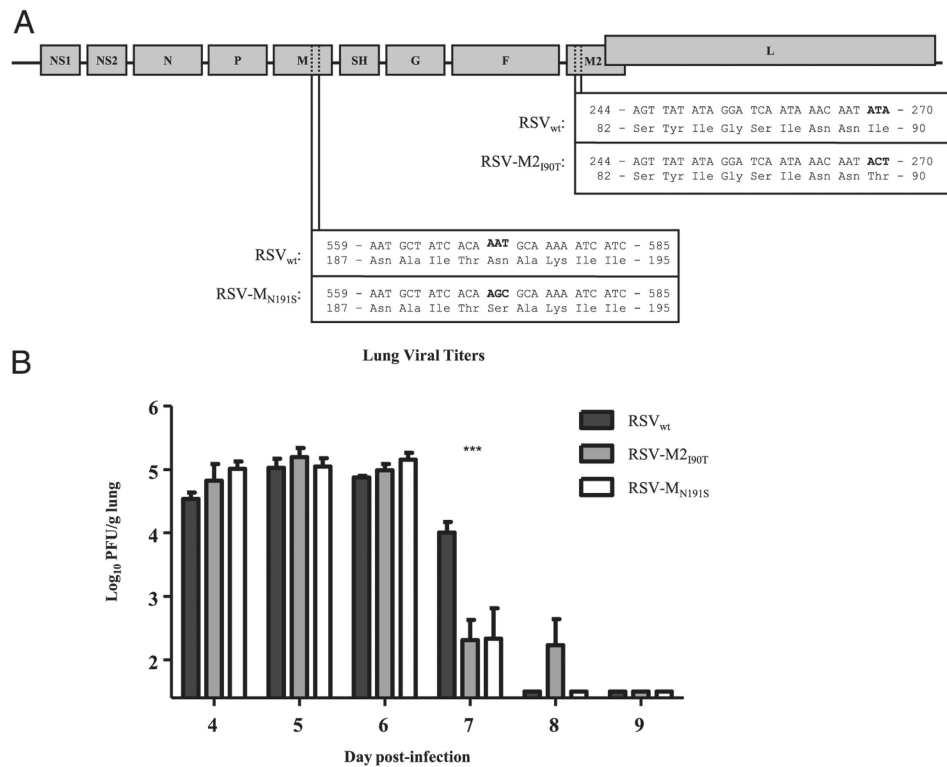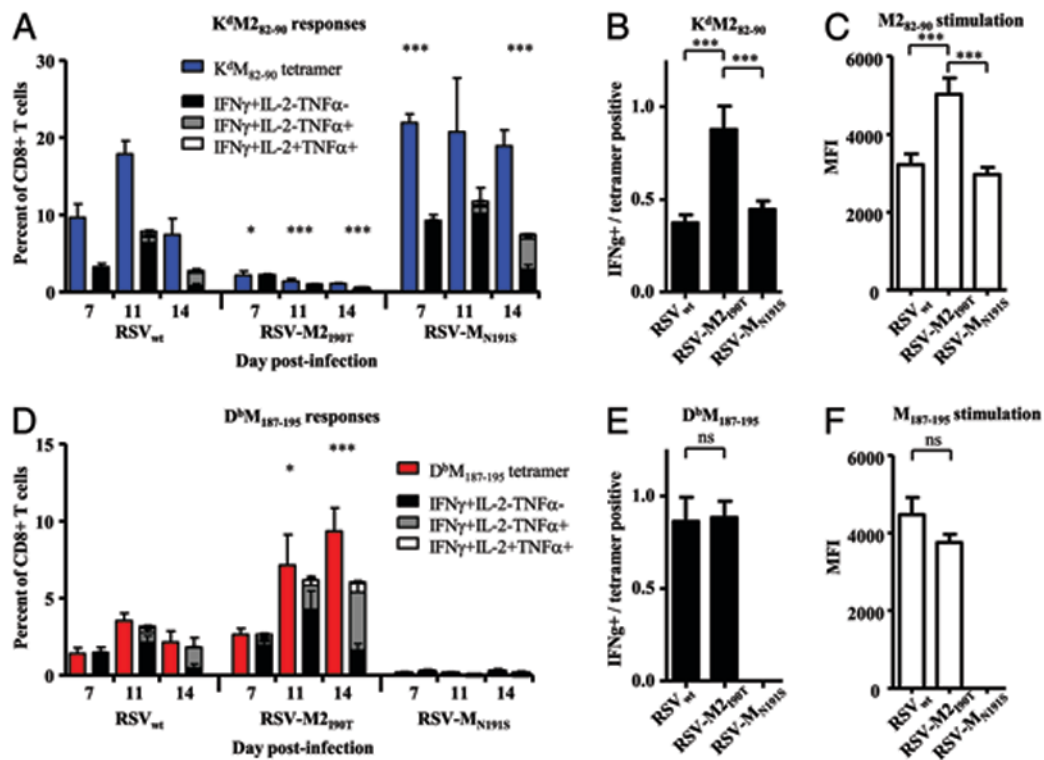
Figure 1.
Live RSV with anchor residue mutations in the K^dM2_{82-90} and $D^bM_{187-195}$ epitopes can be rescued and grow with wt phenotype. A, Single amino acid changes of the RSV genome were made by point mutation in smaller subcloning plasmids prior to introduction into a plasmid containing the entire RSV genome and rescue by reverse genetics (as described in *Materials and Methods*). B, RSV viral titers in the lungs following infection with 3×10^6 PFU of RSV_{wt}, $RSV-M2_{I90T}$, or $RSV-M_{N191S}$ between days 4 and 9 postinfection. ***$p <$ 0.001. Data are representative of two experiments with four to five mice/group and error bars represent the SEM.
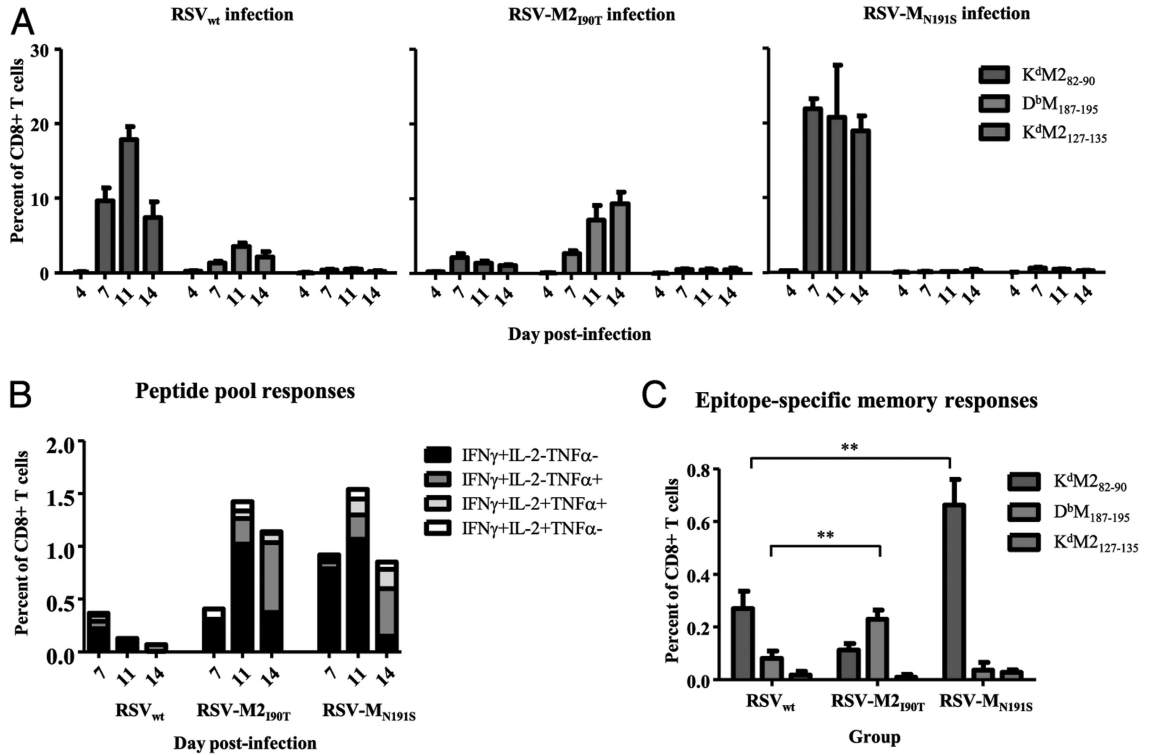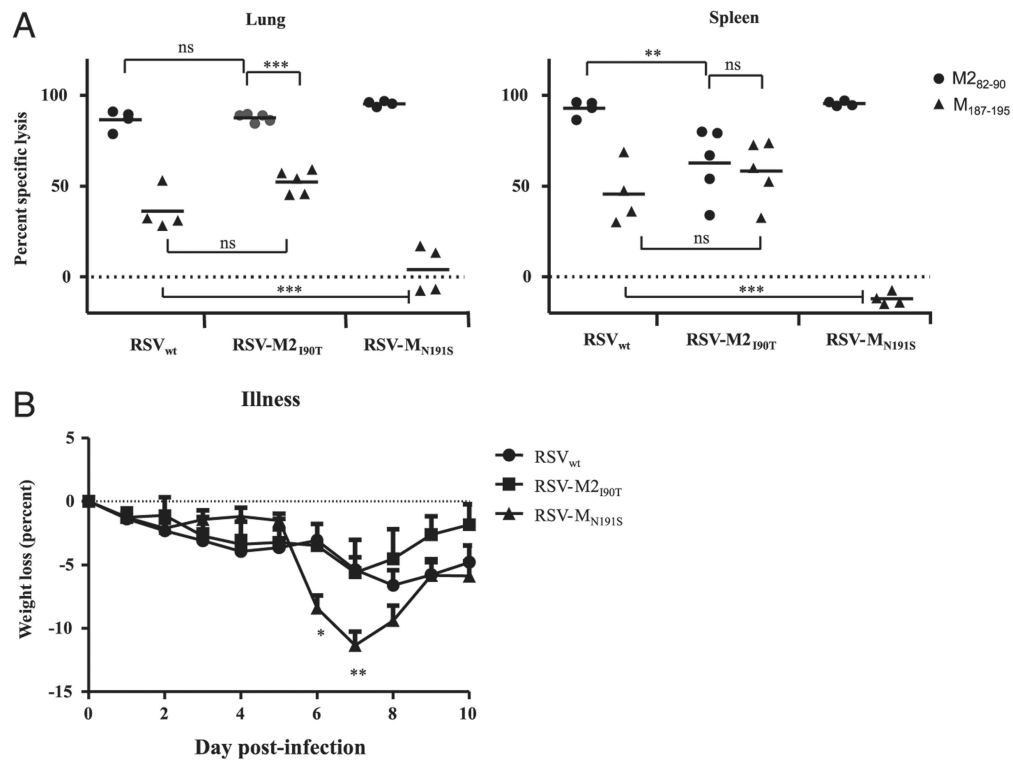
Figure 2.
RSV epitope-specific CD8^{+} T cell responses and memory in mice infected with RSV$_{wt}$, RSV-M2$_{I90T}$, or RSV-M$_{N191S}$. A, Tetramer-positive cells specific for K^{d}M2$_{82-90}$, D^{b}M$_{187-195}$, and K^{d}M2$_{127-135}$ following infection with RSV$_{wt}$, RSV-M2$_{I90T}$, and RSV-M$_{N191S}$. Lymphocytes were isolated from the lungs of infected CB6F1, and stained with tetramers and surface phenotype Abs. Percentages of CD3^{+}CD8^{+} T cells staining with tetramer are shown. B, Intracellular cytokine responses to a pool of minor epitope peptides (F$_{85-93}$, F$_{250-258}$, F$_{433-442}$, N$_{57-64}$, and N$_{360-368}$) in the lungs following infection with RSV$_{wt}$, RSV-M2$_{I90T}$, and RSV-M$_{N191S}$. C, Epitope-specific memory responses in the spleen of mice infected 100 d earlier with RSV$_{wt}$, RSV-M2$_{I90T}$, and RSV-M$_{N191S}$ as determined by staining with tetramer and surface phenotype Abs. $**p < 0.01$. Data are representative of two to three experiments done with four to five mice per group and error bars represent the SEM.

Figure 3.
Quantity versus quality of RSVepitope-specific CD8+ T cell responses in mice infected with RSV$_{wt}$, RSV-M2$_{I90T}$, or RSV-M$_{N191S}$. A, Frequency and cytokine production by K^{d}M2$_{82-90}$-specific CD8+ T cells in the lung following infection with RSV$_{wt}$, RSV-M2$_{I90T}$, or RSV-M$_{N191S}$. Parallel samples were stained with tetramer and incubated with M2$_{82-90}$ peptide for 5 h in the presence of the transport inhibitor monensin. B, Fraction of K^{d}M2$_{82-90}$-specific cells capable of producing cytokine following M2$_{82-90}$ peptide stimulation. C, MFI of IFN-γ–positive cells following peptide stimulation with M2$_{82-90}$ following group gating on cells producing IFN-γ. D, Frequency and cytokine production by D^{b}M$_{187-195}$ epitope-specific CD8+ T cells following infection. E, Fraction of D^{b}M$_{187-195}$-specific cells capable of producing cytokine following M$_{187-195}$ peptide stimulation. F, MFI of IFN-γ–positive cells following peptide stimulation with M$_{187-195}$. *$p < 0.05$ compared with RSV$_{wt}$; ***$p < 0.001$ compared with RSV$_{wt}$. Data are representative of two experiments with four to five mice/group and error bars represent the SEM. ns, no significance.

Figure 4.
In vivo cytotoxicity and illness in mice infected with RSV_{wt}, $RSV-M2_{I90T}$, or $RSV-M_{N191S}$. A, Specific lysis of peptide-pulsed target cells fifteen hours after i.v. transfer into mice infected with RSV_{wt}, $RSV-M2_{I90T}$, and $RSV-M_{N191S}$. Percent specific lysis of cells pulsed with $M2_{82-90}$ (circle) or $M_{187-195}$ (triangle) were calculated by comparison with labeled cells not pulsed with peptide. Cells recovered from naive mice injected with all four cell populations were used to calculate input. **$p < 0.01$; ***$p < 0.001$. ns, no significance. B, Illness in mice infected with RSV_{wt}, $RSV-M2_{I90T}$, and $RSV-M_{N191S}$ was evaluated by weighing mice daily and comparing to their weight at the time of infection (day 0). *$p < 0.05$; **$p < 0.01$.

Table I

CD8$^+$ T cell epitopes described in BALB/c and C57BL/6 mouse strains

Epitopes	Reference	RSV Protein	MHC Restriction	Peptide Sequence[a]
Epitopes described in BALB/c mice (H-2^d)	(26)	M2$_{82-90}$	K^d	SYIGSINNI
	(23)	F$_{85-93}$	K^d	KYKNAVTEL
	(27)	M2$_{127-135}$	K^d	VYNTVISYI
	(24)	F$_{92-106}$	K^d	ELQLLMQSTPPTNNR
	(25)	F$_{249-258}$	K^d	TYMLTNSELL
Epitopes described in C57BL/6 (H-2^b)	(29)	M$_{187-195}$	D^b	NAITNAKII
	(28)	F$_{250-258}$	D^b	YMLTNSELL
	(28)	F$_{433-442}$	D^b	KTFSNGCDYV
	(28)	N$_{57-64}$	K^b	ANHKFTGL
	(28)	N$_{360-368}$	D^b	NGVINYSVL

[a]Anchor residues are shown in bold.