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Enhancement of the CD8⁺ T cell response to a subdominant epitope of respiratory syncytial virus by deletion of an immunodominant epitope

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Abstract

Cytotoxic T lymphocytes are critical for the control of respiratory syncytial virus infection in humans and mice. Recently, we identified a new H-2K^d restricted subdominant epitope in the RSV M2 protein. In this study, we investigated if modification of anchor residues at positions 2 and 9 in the dominant M2_{82–90} epitope in the M2 protein would alter the CTL epitope dominance hierarchy following immunization with plasmid DNA encoding M2 proteins. We showed that immunogenicity of the subdominant epitope M2_{127–135} was enhanced when the anchor residues of the dominant epitope were mutated, suggesting that the immunodominant epitope induces a suppression of response to the subdominant epitope.

Keywords

Respiratory Syncytial Viruses; DNA vaccines; Epitopes; T-Lymphocyte

Introduction

Respiratory syncytial virus causes serious lower respiratory tract illness in infants, young children and the elderly [1–3]. To date, no vaccine has been approved for human use, and serious concerns have been raised about the previous experience with a formalin-inactivated respiratory syncytial virus (RSV) vaccine candidate in which vaccination was associated with enhanced disease [4–6]. Virus-specific cytotoxic T lymphocytes (CTLs) are critical for controlling RSV infection in mice and humans and thus are likely to be an important mediator for protection induced by a successful RSV vaccine. Previously, it has been shown that adoptively-transferred CD8⁺ CTLs can protect BALB/c mice from secondary infection by

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limiting viral replication *in vivo* [7]. However, the same CD8⁺ CTLs can contribute to RSV pathogenesis in the lungs and potentially lethal pulmonary disease in mice when administered in high doses [7]. Therefore, an intricate balance between controlling infection and aggravating disease by RSV-specific CD4⁺ and CD8⁺ T cells likely needs to be established when developing a safe and effective vaccine for humans.

CD8⁺ T cells recognize small antigenic peptides (8–10 amino acids) that are associated with major histocompatibility complex (MHC) class I molecules. For most viruses, although there are a number of potential motifs that are favorable for class I binding, only a handful of dominant epitopes are found. In humans, a number of HLA-restricted RSV T cell epitopes have been found in the nucleocapsid (N) protein, fusion (F) protein, the matrix 2 open reading frame 1 (M2-1) protein and the short hydrophobic (SH) protein [8–10]. In mice, the CD8⁺ T cell responses are highly focused on a few epitopes in the F, glycoprotein (G), matrix (M) and M2-1 proteins, possibly due to more limited breadth of MHC class I molecules in inbred animals [11–15]. A hierarchy in the immunogenicity of these RSV epitopes has been demonstrated [16,17]. Epitopes that induce the most robust CTL responses are termed immunodominant epitopes, while those eliciting lower level or frequency of responses have been termed subdominant epitopes. The mechanisms governing this hierarchy are poorly understood but could be attributed to several factors such as (i) the kinetics and amount of antigen production, (ii) the efficiency of intracellular processing of antigenic peptide, (iii) the binding affinity of peptide to MHC class I and (iv) the T-cell receptor repertoire [18–22]. CTL responses from immunodominant epitopes have been reported to directly suppress the development of CTLs from subdominant epitopes through interferon- γ (IFN- γ) [23]. In individuals infected with human immunodeficiency virus (HIV), CTL responses targeting subdominant epitopes have been found to contribute to control of virus replication and escape [24,25]. However, vaccine constructs that target multiple epitopes with different degrees of dominance failed to alter the inherited immunodominance hierarchy in animals [26].

We previously identified a RSV-specific, H-2K^d-restricted subdominant epitope in the M2-1 protein (M2_{127–135}: VYNTVISYI) in BALB/c mice that is present in the same protein as the most dominant epitope in the virus for BALB/c mice (M2_{82–90}: SYIGSINNI) [27]. We found that there is a tissue-specific regulation of CD8⁺ T lymphocytes immunodominance in RSV infection that affects the ratio of cells specific for these two epitopes. In the current study, we sought to determine if the immunodominance hierarchy could be manipulated by vaccination with altered immunogens. We examined the immunogenicity of a DNA vaccine where the anchor residues of the dominant epitope (M2_{82–90}: SYIGSINNI) were mutated. A comparison of the responses by DNA vaccination to these two epitopes revealed a consistent immunodominance hierarchy as seen in infection model. The loss of the dominant T cell response from M2 mutant DNA immunization in BALB/c mice was compensated for by increased induction of subdominant responses. Furthermore, these subdominant T cells reduced viral replication in the vaccinated mice after RSV challenge. Taken together, this study shows promise for the approach of manipulating epitope hierarchy by depletion of an immunodominant epitope, and has implications for a rationale approach to T cell vaccine design.

Materials and Methods

Mice

Respiratory pathogen-free 6–8 week old female BALB/c (H-2^d) mice were purchased from Harlan Sprague-Dawley Laboratory (Indianapolis, IN, USA). We adhered to the *Guide for the Care and Use of Laboratory Animals* of the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council in conducting the research described in this paper. All experiments were reviewed and approved by the

Vanderbilt Institutional Animal Care and Use Committee. Animals were maintained in microisolator cages throughout the studies.

Cells and Virus

HEp-2 cells (ATCC no. CCL-23) were maintained in Opti-MEM I medium (Invitrogen) supplemented with 4 mM glutamine, 5 µg/ml amphotericin B, 50 µg/ml gentamicin, and 2% fetal bovine serum. The RSV wild-type strain A2 was grown in HEp-2 cell monolayer cultures, titrated, and stored in aliquots at -80°C until use.

Peptide Design

Peptides for use in MHC-binding affinity assays were synthesized as previously described [27]. The peptides tested were based on the H-2^d-restricted immunodominant peptide in the wild-type sequence of the RSV strain A2, a peptide spanning amino acids 82–90 and designated here M2_{82–90} (SYIGSINNI), and a peptide spanning amino acids 127–135 and designated here M2_{127–135} (RVYNTVISY). Variant peptides and the rationale for them are shown in Table 1.

Peptide Synthesis

Peptides were derivatized with a monodisperse PEG₆₈₅ linker (Quanta BioDesign), for improved solubility, using a standard coupling procedure. m-dPEG₆₈₅ NHS ester (4 eq) and HOBt (4 eq) were dissolved in DMF and added to the N-Fmoc deprotected peptidyl resin. The coupling reaction was carried out overnight and its completeness was verified by the Kaiser test. Purified peptides were lyophilized (Labconco Freezezone 4.5) and stored at -80°C until use. The peptides were synthesized and high-performance liquid chromatography-purified to more than 95% purity.

DNA vaccine design and preparation

We generated a plasmid DNA encoding the M2-1 protein of RSV wild-type strain A2 by cloning a sequence-optimized M2-1 cDNA into a mammalian cell expression vector that uses the CMV promoter. Gene optimization of the M2-1 open reading frame (605 bp) was performed using GeneOptimizer™ Software (GENEART GmbH, Regensburg, Germany). A codon-modified M2-1 gene was synthesized by GENEART. To generate the final wild-type DNA vaccine construct, the optimized M2-1 gene was subcloned between XhoI and BamHI restriction sites of pcDNA3.1 (Invitrogen). The wild-type plasmid (designated here M2-wt) contained both the H-2^d-restricted immunodominant peptide spanning amino acids 82–90 (SYIGSINNI) and the subdominant epitope peptide spanning amino acids 127–135 (RVYNTVISY). A second DNA vaccine (designated here M2-mut) was generated that specified an altered sequence in the 82–90 immunodominant epitope region that contained two point mutations (Y83R and I90T) that eliminated the predicted anchor residues that are required for K^d binding for that epitope, while the subdominant epitope sequence was left unaltered. Plasmids encoding the M2-wt and M2-mut sequences were purified using the Endofree plasmid Giga kit (Qiagen, Hilden, Germany).

DNA immunization

Six-week-old female BALB/c mice were anesthetized with inhaled isoflurane (Aerrane, Baxter Healthcare Corporation, Deerfield, IL). Mice were immunized intramuscularly with 100 µg of RSV M2-wt or M2-mut plasmid DNA formulated in PBS divided into two 50 µg doses, one injected into each quadriceps. Immunization was performed at three time points, on day 0, day 14, and day 21.

RMA-S stabilization assay

RMA-S cells expressing K^d were kindly provided by Dr. E. Pamer (Memorial Sloan Kettering Cancer Center, New York, NY). The RMA stabilization assay was performed as described previously [19]. RMA-S cells were maintained in complete RPMI 1640 medium supplemented with 10% FBS, L-glutamine, 2-mercaptoethanol, HEPES, and gentamicin. RMA-S-K^d cells (10⁶) were maintained at 26 °C for 18–24 h, pulsed with indicated amounts of peptide in duplicate for 45 min at 26 °C in a 5% CO₂ atmosphere, and transferred to 37 °C for an additional 3 h. Cells were stained with biotinylated anti-K^d mAb (clone SF1-1,1, Pharmingen, San Jose, CA) followed by PE-conjugated streptavidin (Pharmingen), washed twice with FACS buffer, and analyzed by flow cytometry. The results were expressed as the mean fluorescence intensity (MFI) ratio. Percent MFI increase was calculated as follows: Percent MFI increase = (MFI with the given peptide-MFI without peptide)/(MFI without peptide) × 100.

Preparation of splenocytes

Mice were sacrificed by CO₂ inhalation at varying time points, as indicated in the figures. The spleen was removed from each animal and placed separately into complete RPMI supplemented with 10% FBS, L-glutamine, 2-mercaptoethanol, HEPES, and gentamicin (designated R10 medium). The tissues were minced and ground through a sterile steel mesh to obtain a single-cell suspension. Cells were treated with red blood cell lysing buffer (Sigma-Aldrich, St-Louis, MO). Cells were counted and resuspended at the stated cell concentration for the appropriate *in vitro* assay.

ELISpot assay

96 well plates (MAIPSWU 10, Millipore, Bedford, MA) were coated with 10 µg/ml of anti-IFN-γ monoclonal antibody (clone A18, Mabtech, Stockholm, Sweden) in PBS (without Ca²⁺ or Mg²⁺) at 4 °C overnight. Plates then were washed with sterile PBS three times and blocked with 10% RPMI for at least 2 hours at room temperature. Peptides were added directly to wells in a volume of 50 µl, and then freshly isolated splenocytes were added at a concentration of 10⁵ cells/well in 50 µl of R10 medium. The final concentration of the peptides in the screening assay was 10 µM. The plates were incubated for 18–20 h at 37 °C in 5% CO₂. The plates then were washed, labeled with 2 µg/ml biotinylated anti-IFN-γ mAb (clone R4-6A2, Mabtech) in PBS, and incubated at room temperature for 3 h. After additional washes, avidin-peroxidase complex (Vector Laboratories, Burlingame, CA) was added to each well in PBS for 1 h at RT. The plates were washed, and IFN-γ producing cells were detected after a 4 min color reaction using 100 µl of AEC substrate (20 mg of 3-amino-9-ethylcarbazol [Sigma-Aldrich]) dissolved in 2.5 ml of dimethylformamide, diluted 1:20 in 47.5 ml of sodium-acetate buffer + 25 µl 30% H₂O₂. IFN-γ-producing cells were counted using an automated ELISpot reader system and ImmunoSpot 4 software (Cellular Technology Ltd, Cleveland, OH). Results were expressed as the number of spot-forming cells (SFC) per 10⁶ input cells.

Intracellular Cytokine Staining (ICS) Assay and Tetramer Staining

To enumerate the number of IFN-γ-producing cells, intracellular staining was performed as previously described [28]. In brief, freshly isolated splenocytes (2 × 10⁶) were left untreated or stimulated with individual peptides (1 µg/sample) or treated with PMA (10 ng/ml) and ionomycin (500 ng/ml) and costimulatory monoclonal antibodies anti-CD28 and anti-CD49d for 6 h at 37 °C in 5% CO₂. Brefeldin A (10 µg/ml; Sigma) was added during the culture period to facilitate intracellular cytokine accumulation. Cell surface staining was performed followed by intracellular cytokine staining using the Cytotfix/Cytoperm kit (BD Pharmingen, San Diego, CA) in accordance with the manufacturer's protocol. For tetramer analysis, freshly isolated cells (2 × 10⁶) in R10 medium were incubated with pre-titered, optimal amounts of H-2K^d M2 tetramers for 1h on ice followed by surface staining for CD3, CD4, and CD8.

The following antibodies were obtained from BD Pharmingen: anti-CD3 FITC, anti-CD4 PE-Cy7, anti-CD8 Cy7-allophycocyanin, and anti-IFN- γ PE. Tetramers were obtained from Beckman Coulter. The peptide epitopes used in these tetramers were SYIGSINNI (M2₈₂₋₉₀) and VYNTVISYI (M2₁₂₇₋₁₃₅). Flow cytometry was performed using an LSRII cytometer (BD Immunocytometry Systems). Data analysis was performed using FlowJo software version 8.3 (Tree Star, San Carlos, CA).

Challenge, tissue collection, and plaque assay

Mice were challenged intranasally (i.n) with 10⁶ PFU RSV strain A2 7 days after the third immunization. On day 4 after challenge, mice were sacrificed by CO₂ inhalation. The lungs and nasal turbinates were harvested separately for virus quantification by plaque assay. Lung tissues were ground using a glass homogenizer in 3 ml of HBSS (Hanks Balanced Salt Solution) medium, and nasal turbinates were homogenized in 3 ml by grinding in a cold mortar and pestle with sterile sand. Homogenates were clarified by centrifugation in a tabletop centrifuge at 2,000 rpm at 4 °C for 10 min, and supernatants were frozen in cryovials at -80 °C until use. To determine RSV titers, 100 μ l of 10-fold serial dilutions of the homogenates was added to confluent HEP-2 cell monolayer cultures in duplicate and cultured for 4 days under a semisolid methylcellulose overlay medium, followed by fixation with 80% cold methanol. Virus plaques were visualized by staining with immunoperoxidase using a cocktail of three anti-F protein mouse monoclonal antibodies.

Statistical analysis

Significant differences among groups were determined using Student *t*-test.

RESULTS

Binding affinity of MHC class I to peptides with altered anchor residues at position 2 and/or position 9

Nine amino acid long peptides were synthesized containing the wild-type M2₈₂₋₉₀ sequence (M2₈₂₋₉₀) or alternate sequences lacking either the canonical anchor residues at position 2 (M2₈₂₋₉₀ Δ P2), position 9 (M2₈₂₋₉₀ Δ P9) or both position 2 and 9 (M2₈₂₋₉₀ Δ P2 Δ P9) as shown in Table 1. We tested the relative affinity of each of the peptides for binding to the MHC class I molecule using the RMA-S cell MHC stabilization assay. A hierarchy of affinity was observed as evidenced by graded mean fluorescence intensities at varying intensities that differed for the various peptides. Cell surface MHC was not detected in the absence of peptide, as expected. The M2₈₂₋₉₀ peptide exhibited the highest affinity, the M2₈₂₋₉₀ Δ P9 peptide showed about 10-fold reduced affinity, and the M2₈₂₋₉₀ Δ P2 showed about 100-fold lower affinity to MHC class I molecules. Cell surface MHC class I binding was not detected for M2₈₂₋₉₀ Δ P2 Δ P9 peptide at any concentration tested (Figure 1).

T cell responses to mutated M2 genes *in vivo*

Next we constructed complete cDNA copies of the M2 gene with or without the immunodominant M2₈₂₋₉₀ epitope, as shown in Figure 2. The wild-type M2 gene was prepared as a DNA vaccine construct, as was a variant lacking both the canonical anchor residues at position 2 and 9, designated M2-mut. We immunized BALB/c mice with one of the DNA vaccine constructs two or three times and evaluated the induction of peptide-specific interferon- γ -secreting cells in the spleens of these vaccinated mice by ELISPOT. We previously showed that 15-mer peptides containing the immunodominant or subdominant epitopes worked better as reagents in ELISPOT or intracellular cytokine assays than the defined epitopes, which were of shorter length. Here, we used peptide M2₇₇₋₉₂ or peptide M2₈₁₋₉₆ as the dominant M2₈₂₋₉₀ epitope and peptide M2₁₂₁₋₁₃₆ or peptide M2₁₂₅₋₁₄₀ as the subdominant M2₁₂₇₋₁₃₅

epitope in our ELISPOT assay. Both peptides from each individual M2 epitope gave comparable results in terms of the numbers of interferon- γ -secreting cells induced (Figure 3). Empty vector DNA immunization did not induce a significant response (Figure 3A and D). As expected, immunization with the M2-wt induced a robust response for the immunodominant M2₈₂₋₉₀ epitope, with over 200 spot forming cells per million splenocytes and a response to the subdominant epitope M2₁₂₇₋₁₃₅ that was about four-fold lower (Figure 3B), as we have reported previously. Surprisingly, a T cell response was not detected in animals immunized with the M2-mut gene two weeks after vaccination (Figure 3C).

Following a third DNA immunization, responses to both immunodominant and subdominant epitopes were boosted, but the relative magnitudes remained comparable (Figure 3E). As expected, immunization with the M2-mut vaccine lacking the M2₈₂₋₉₀ epitope did not induce T cells to that epitope (Fig 3F). Remarkably, however, the M2-mut DNA vaccine induced a significantly enhanced T cell response to the subdominant epitope after three immunizations, with more than 400 spot forming cells per million cells detected with either of the peptides containing the subdominant epitope (Figure 3F). It should be noted that this magnitude of response was comparable to that mediated by the immunodominant epitope following M2-wt gene vaccination (Figure 3E).

Immunogenicity of mutated M2 genes measured by intracellular cytokine staining

There was the theoretical possibility that the interferon- γ -secreting cells induced by vaccination that were detected by ELISPOT could have been induced by cells other than class I MHC-restricted CD8+ T cells. Therefore, we also used an alternate technique to assess immunogenicity of the vaccine variants that defined the cell source of interferon- γ , namely flow cytometry-based intracellular cytokine assay (Figure 4). Splenocytes tested with a control peptide revealed that immunization with M2-wt or M2-mut vector was associated with a low background of non-specific interferon- γ -secreting CD8+ T cells (0.13 % in both cases). PMA/ionomycin stimulation was used as a maximal stimulus, yielding over 30% of cells expressing interferon- γ . Immunization with the M2-wt DNA vaccine induced a high percentage of CD8+ T cells reactive with the immunodominant epitope (2.6 – 2.8 %), while vaccination with the M2-mut construct lacking this epitope did not (0.1 – 0.31 %). The M2-wt vaccine induced lower responses to the subdominant epitope (0.53 – 0.69 %). The approximate 4:1 ratio of immunodominant to subdominant reactivity consistently seen with this technique was nearly identical to that detected by ELISPOT.

Frequency of M2-specific CD8+ T cells in M2-mut-immunized mice

We also assessed the number of M2-specific CD8+ T cells in mice immunized with M2-wt or M2-mut DNA using M2₈₂₋₉₀ and M2₁₂₇₋₁₃₅ MHC tetramers. As expected, in mice vaccinated with M2-wt DNA, there was a higher frequency of CD8+ T cells specific for the M2₈₂₋₉₀ dominant epitope (1.2%) compared to the M2₁₂₇₋₁₃₅ subdominant epitope (only 0.05%). In contrast, mice immunized with M2-mut DNA had a higher frequency of the subdominant epitope-specific CD8+ T cells (1.3%) versus the M2₈₂₋₉₀ specific CD8+ T cells (0.2%). Surprisingly, the frequency of M2₈₂₋₉₀-specific T cells in M2-wt immunized mice was comparable to the frequency of M2₁₂₇₋₁₃₅-specific T cells in M2-mut immunized animals (Figure 5).

Virus clearance in M2-mut DNA immunized mice

Given the change of epitope hierarchy in M2-mut DNA immunized animals, we were interested to see how this alteration affected viral replication *in vivo*. We challenged mice with RSV that were immunized previously with M2-wt or M2-mut DNA and measured viral titers in the lungs and nasal turbinates of these animals 4 days post-infection. Animals that were mock vaccinated with PBS or vaccinated with an empty vector exhibited high levels of viral titers in both the

lungs (5.2–5.3 log₁₀ PFU per gram of tissue) and nasal turbinates (4.7–4.8 log₁₀ PFU/gram of tissue). Mice that were vaccinated with M2-wt DNA vaccine exhibited a 100-fold or 20-fold reduction in viral titer in the lungs or nasal turbinates respectively. A similar reduction was seen with mice vaccinated with the M2-mut DNA vaccine in the nasal turbinates, although there was only a 5-fold reduction in viral titer in the lungs (Table 2). Nonetheless, virus clearance was observed in both M2-wt and M2-mut DNA vaccinated group compared to control groups.

DISCUSSION

DNA vaccination has been proven to be a successful immunization scheme in animal models to induce CD8+ CTLs for protection. Most gene-based T-cell vaccines have focused on inducing responses to a few immunodominant epitopes [29–31]. We described previously a subdominant epitope in the RSV M2-1 protein (M2_{127–135}) and showed that there is a tissue-specific regulation of the ratio of dominant epitope to subdominant epitope CTLs in various organs in mice during RSV infection. Other models have also shown that overall immunodominance patterns in antiviral CD8+ T cell responses can be changed by prolonged antigen stimulation [21,32] or altering MHC alleles in transgenic mice [33]. In this study, we examined a DNA vaccine that lacked the M2-1 dominant epitope *in vivo* and showed that it could induce compensatory cellular responses from the subdominant epitope in the same viral protein.

MHC class I molecules display diverse peptides for T cell receptor recognition. Peptide binding onto allele-specific MHC class I molecules usually requires specific amino acid side-chain residues present at position 2 and 9 of the peptide. Binding of these residues into the MHC pocket stabilizes the MHC-peptide complex on the cell surface for T cell interaction. We mutated the amino acids at positions 2 and 9 of the immunodominant peptide M2_{82–90} and measured the affinity of the peptide to K^d MHC-I molecules. Reduced peptide binding affinity was observed for peptides with a single mutation at either position 2 or 9. Direct binding could not be detected for a peptide with both of the anchor residues mutated, at any concentration tested. These data suggested that these mutations likely completely abolished loading of these peptides onto the MHC-I molecules on professional antigen presenting cells *in vivo*.

We have shown previously that the ratio of responses to the dominant epitope and the subdominant epitope in the M2-1 protein varies in different anatomic sites during RSV infection [27]. We investigated the responses of a DNA vaccine encoding both the dominant and subdominant epitopes in the RSV M2-1 protein (M2-wt). Response to the dominant epitope was seen after two DNA immunizations and the ratio of dominant to subdominant responses was maintained at a 3:1 ratio, as we have reported previously following RSV infection [27]. We also compared the responses in mice that were vaccinated with DNA lacking the M2 dominant epitope. We observed a delayed kinetics in the induction of M2_{127–135}-specific T cells in M2-mut immunized mice when compared to the generation of M2_{82–90}-specific T cells in M2-wt vaccinated animals. Only after the third immunization did we see the induction of subdominant epitope-specific T cells. However, we do not yet understand the mechanism by which epitope dominance affects the kinetics of induction of T cells *in vivo*. We speculate that this interesting finding may in part be attributed to the 300-fold lower affinity of binding to MHC class I molecules for the M2_{127–135} peptide than for the M2_{82–90} peptide, thus altering the MHC-I binding process and presentation. Although the responses were delayed, the numbers of M2_{127–135} tetramer-specific CD8+ T cells induced in M2-mut vaccinated mice were similar to the number of M2_{82–90} tetramer-specific CD8+ T cells induced in M2-wt vaccinated animals. This finding may indicate a compensatory effect of T cells responding to the subdominant epitope when the dominant epitope is deleted.

In addition, we performed intracellular cytokine staining to determine responses from splenocytes in vaccinated animals. When splenocytes from M2-wt immunized mice were stimulated with M2 dominant or subdominant peptides, the frequency of M2₈₂₋₉₀-specific, interferon- γ secreting splenocytes was about three-fold higher than M2₁₂₇₋₁₃₅-specific cells, consistent with the ELISPOT data as well as our previous observations [27]. In the case of M2-mut vaccinated animals, the frequency of M2₁₂₇₋₁₃₅-specific T cells doubled while the frequency of M2₈₂₋₉₀ was markedly reduced. We showed that the induction of subdominant epitope-specific T cells was delayed in our vaccination model. An interesting feature of the data is that the frequency of M2₁₂₇₋₁₃₅-specific T cells from M2-mut animals was slightly higher than the frequency of M2₈₂₋₉₀-specific T cells from M2-wt animals in this assay. It is possible that these CD8⁺ T cells responding to the subdominant epitope were functionally different than those responding to the dominant epitope in M2. It has been shown that subdominant epitopes from lymphocytic choriomeningitis virus (LCMV) induced protective CD8⁺ T cells populations with differing cytolytic activities and were found to be associated with the kinetics and epitope specificities of the T cells [34]. In addition, while this manuscript was in preparation, Kreml *et al.* reported a recombinant RSV infection approach to demonstrate that cytolytic activity of M2₁₂₇₋₁₃₅-specific T cells was somewhat greater in mice infected with a recombinant RSV containing a mutation in the M2₈₂₋₉₀ epitope compared to those infected with wild-type virus when BAL cells were stimulated with M2₁₂₇₋₁₃₅ peptide *in vitro* [35].

Viral replication also was assessed to determine if immunization with M2-mut DNA could reduce RSV titers in the lungs after wild-type virus challenge. Previously, it was reported that a recombinant vaccinia virus encoding M2 induced M2₈₂₋₉₀-specific cytotoxic T cells and those cells were the sole mediators of protection in immunized animals [36]. In our model, the viral titers in the lungs and the nasal turbinates were reduced in M2-mut immunized animals, but the extent of reduction was not as significant as those vaccinated with M2-wt. The lack of complete protection may be related to the immunization method or the route of immunization. Intranasal live virus infection or vaccination induces both systemic and mucosal immune responses, which could offer a significant advantage over intramuscular DNA immunization that induces principally a systemic immune response. In addition, live virus infection in general produces more antigen and more robust immune responses than DNA vaccination. Nonetheless, DNA immunization with RSV M2 plasmids is a useful tool in this study because the data reveal that immunization with M2 protein with deletion of an immunodominant epitope reduced viral titers in both the lungs and nasal turbinates of vaccinated animals.

In conclusion, this study demonstrated that a CTL epitope hierarchy could be manipulated by deletion of an immunodominant epitope in a single protein DNA vaccine. In the RSV model, the subdominant epitope, M2₁₂₇₋₁₃₅, can serve as an important epitope in the absence of the otherwise immunodominant epitope, M2₈₂₋₉₀. However, the functionality and kinetics of T cells responding to a subdominant epitope may be different than those responding to a dominant epitope. Taken together, our findings have implications for understanding the immune response to RSV infection or vaccination and provide insight into methods for the rational design of vaccines that induce CD8⁺ T cells.

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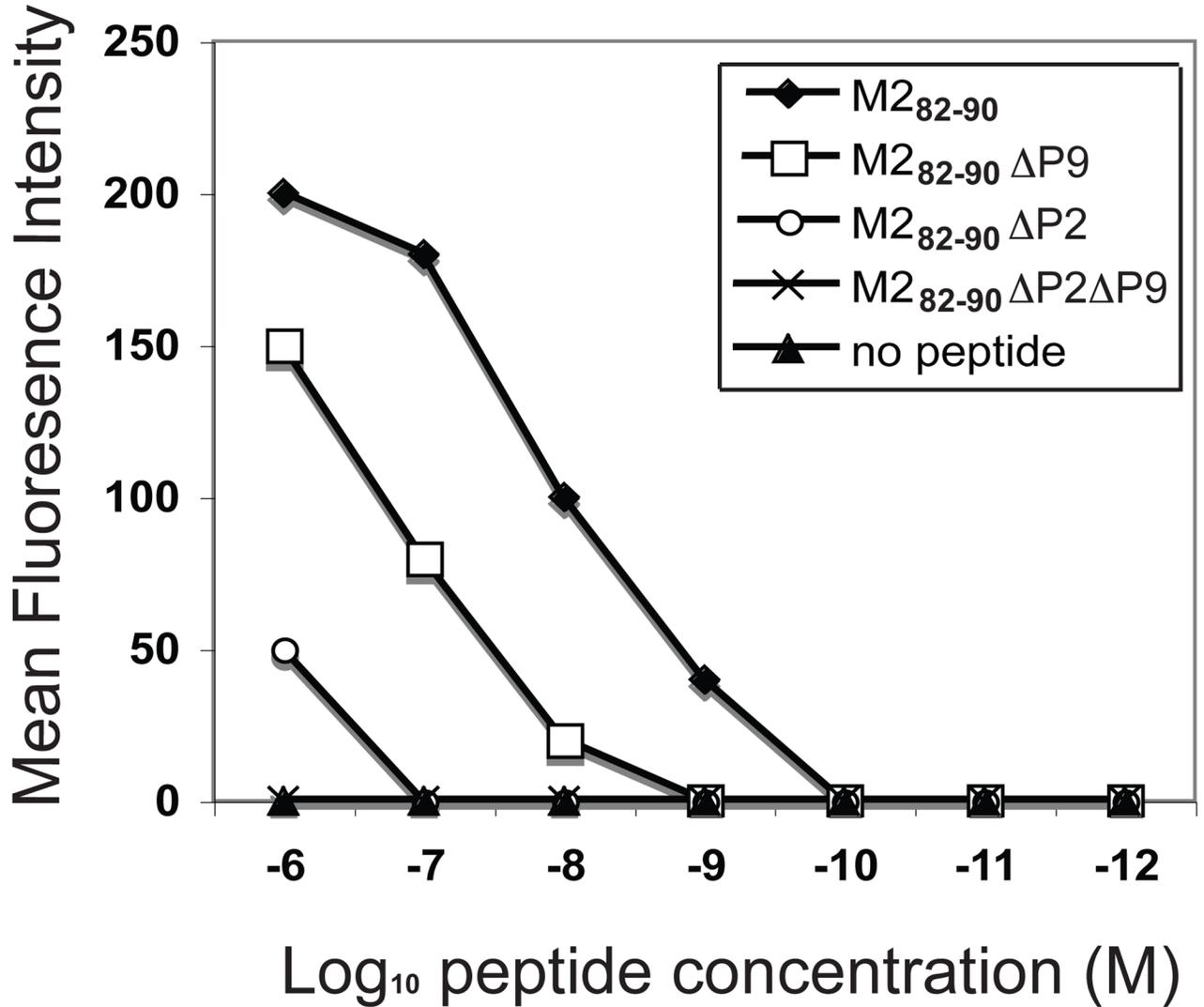


Figure 1.

Binding of wild-type peptides or peptides mutated at H2-K^d anchor residues. RMA-S-K^d cells were incubated at 26 °C, and mixed with peptides at various concentrations. Binding of M2₈₂₋₉₀ (SYIGSINNI) or mutated peptides, M2₈₂₋₉₀ΔP2 (SRIGSINNI), M2₈₂₋₉₀ΔP9 (SYIGSINNT), and M2₈₂₋₉₀ΔP2ΔP9 (SRIGSINNT) to H2-K^d molecules was compared with that of the no-peptide control. The mean fluorescence intensity determined by FACS analysis is plotted against peptide concentration. The results shown represent the data from a single representative experiment, which was one of three with similar results.



Figure 2.
 Schematic diagram of the M2-wt or M2-mut plasmid DNAs. The M2-mut plasmid contains two substituted amino acids, Y83R and I90T.

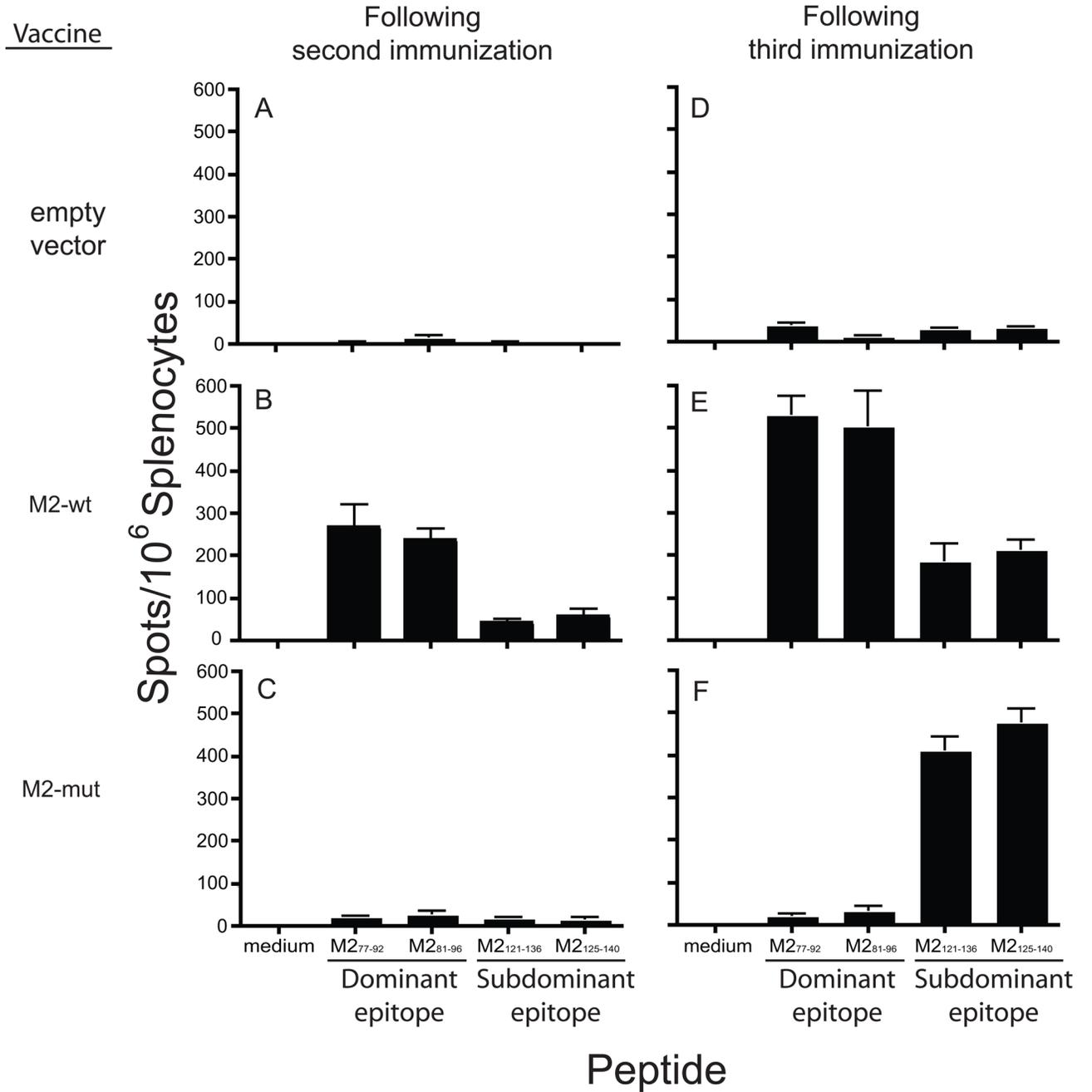


Figure 3. Frequencies of RSV M2-1 dominant and subdominant epitope-specific IFN- γ secreting mouse splenocytes after DNA immunization. BALB/c mice were immunized muscularly with 100 μ g of empty vector (A and D) or DNA encoding either M2-wt protein (B and E) or M2-mut protein (C and F). Splenocytes were harvested 6 days after the second immunization (day 14, B and C) or third immunization (day 21, E and F) and stimulated with peptides containing the dominant M2₈₂₋₉₀ epitope (peptide M2₇₇₋₉₂ and M2₈₁₋₉₆) or peptides containing the subdominant M₁₂₇₋₁₃₅ epitope (peptide M2₁₂₁₋₁₃₆ and M2₁₂₅₋₁₄₀). Data are represented as mean \pm SEM.

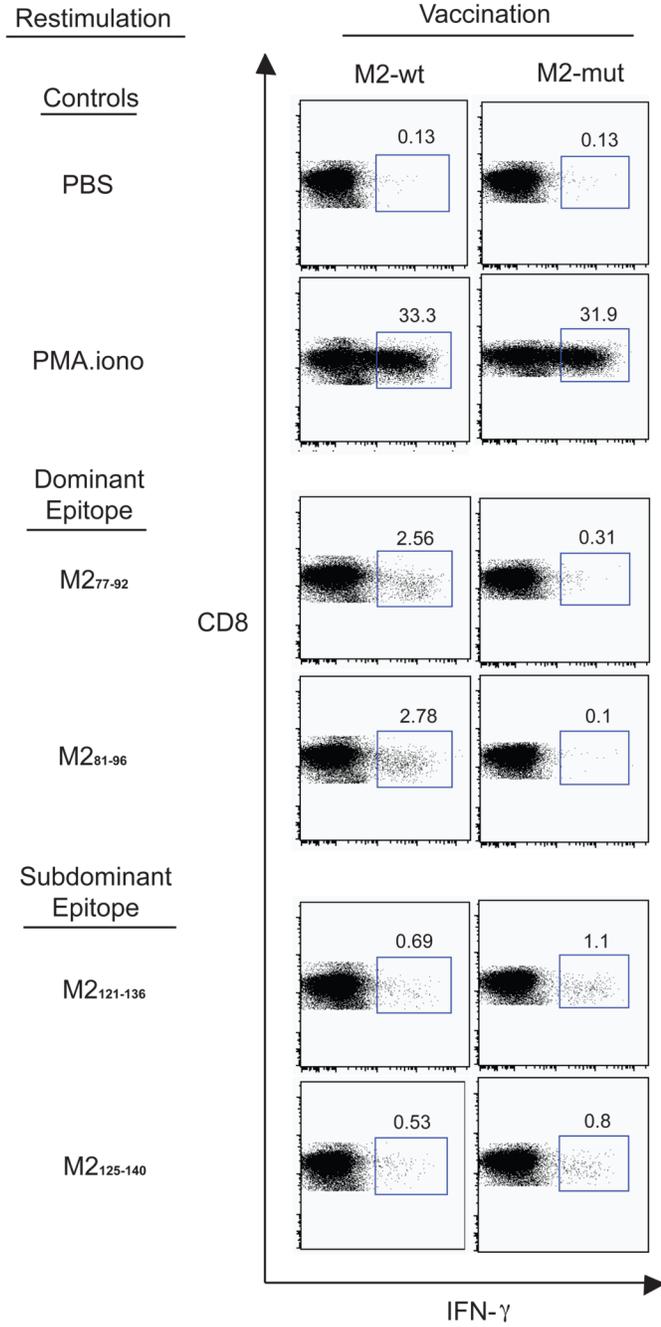


Figure 4. Analysis of peptide-reactive CD8⁺ T lymphocytes 6 days after the third DNA immunization. Splenocytes were incubated with 1 μg/sample of an M2₈₂₋₉₀ containing peptide (peptide M2₇₇₋₉₂ or M2₈₁₋₉₆) or M2₁₂₇₋₁₃₅ containing peptide (peptide M2₁₂₁₋₁₃₆ or M2₁₂₅₋₁₄₀) in an intracellular cytokine staining assay for IFN-γ. The dot plots represent the production of IFN-γ from one of the three representative experiments with similar results. Panels in the left column show results for splenocytes obtained from mice immunized with M2-mut DNA. Panels in the right column show results for splenocytes obtained from mice immunized with M2-wt DNA.

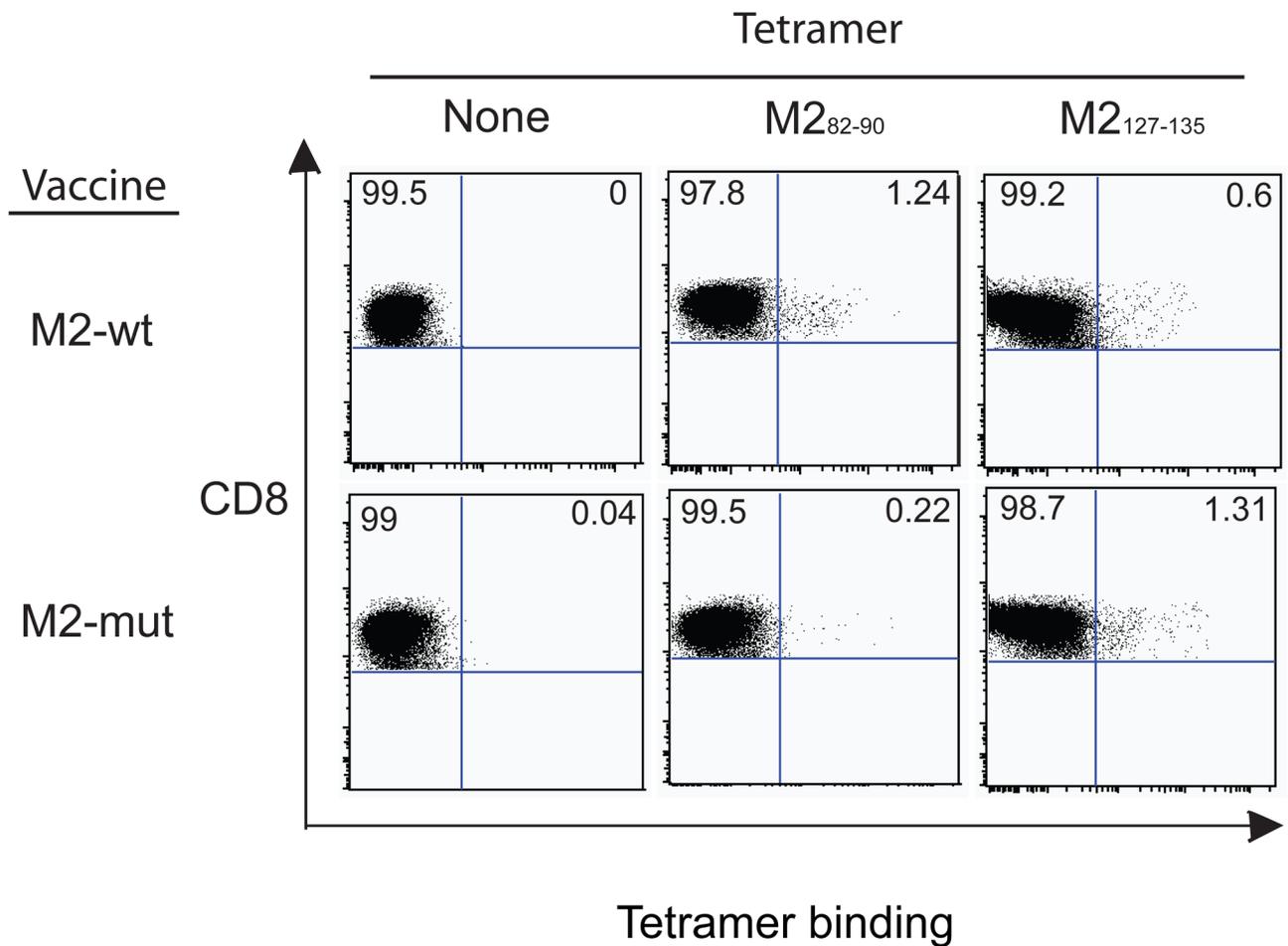


Figure 5.

Direct enumeration of M2₈₂₋₉₀ or M2₁₂₇₋₁₃₅ epitope-specific CD8⁺ T lymphocytes. BALB/c mice were immunized with M2-wt DNA or M2-mut DNA. Splenocytes were harvested 6 days following the third immunization and were analyzed unstained, stained with M2₈₂₋₉₀ tetramer or M2₁₂₇₋₁₃₅ tetramer (B and D). Dot plots represent results for 20,000 CD3⁺/CD8⁺ T lymphocytes. Numbers shown in the rectangular gate correspond to the percentages of CD8⁺ lymphocytes that were tetramer-positive. Data shown are representative of three experiments.

Table 1

Peptide sequences used in these studies

Peptide designation	Sequence	Rationale
M2 ₈₂₋₉₀	SYIGSINNI	The wild-type epitope spanning amino acids 82–90
M2 ₈₂₋₉₀ ΔP2	SRIGSINNI	Alteration of the anchor residue at position 2 (Y83R)
M2 ₈₂₋₉₀ ΔP9	SYIGSINNT	Alteration of the anchor residue at position 9 (I90T)
M2 ₈₂₋₉₀ ΔP2ΔP9	SRIGSINNT	Alteration of both anchor residues (Y83R and I90T)

Table 2

DNA immunization with a vector encoding RSV M2 protein lacking the immunodominant CTL epitope induces significant but reduced protection compared to immunization with the wild-type M2 gene^a

Material used for immunization	Virus titer in the indicated tissue(log ₁₀ PFU/g tissue)	
	Lung	Nasal turbinates
PBS	5.3 ± 0.1	4.8 ± 0.1
pcDNA3.1 (empty vector)	5.2 ± 0.04 ^b	4.7 ± 0.09 ^b
M2-wt DNA vaccine	3.2 ± 0.4 ^c	3.4 ± 0.3 ^d
M2-Y83R_I90T DNA vaccine	4.6 ± 0.1 ^c	3.7 ± 0.4 ^d

^a BALB/c mice (n=5) were immunized with one of the immunogens at day 0, 14 and 21, and challenged with live RSV on day 28 (day 7 after the third immunization, the expected peak day of CTL activity). Lung titers were determined on day 4 following challenge (the expected peak day of viral replication).

^b No significant difference from values obtained for mice immunized with PBS. (P≥0.05)

^c Significantly different from values obtained for mice immunized with PBS (P<0.01).

^d Significantly different from values obtained for mice immunized with PBS (P<0.02).