T Cell Receptor Clonotype Influences Epitope Hierarchy in the CD8+ T Cell Response to Respiratory Syncytial Virus Infection†‡§

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CD8+ T cell responses are important for recognizing and resolving viral infections. To better understand the selection and hierarchy of virus-specific T cell responses, we compared the T cell receptor (TCR) clonotype in parent and hybrid strains of respiratory syncytial virus-infected mice. K4M282–90 (SYIGSIINII) in BALB/c and D6M187–195 (NAITNAKII) in C57BI/6 are both dominant epitopes in parent strains but assume a distinct hierarchy, with K4M282–90 dominant to D6M187–195 in hybrid C57F1/J mice. The dominant K4M282–90 response is relatively public and is restricted primarily to the highly prevalent Vβ13.2 in BALB/c and hybrid mice, whereas D6M187–195 responses in C57BL/6 mice are relatively private and involve multiple Vβ subtypes, some of which are lost in hybrids. A significant frequency of TCR CDR3 sequences in the D6M187–195 response have a distinct “(D/E)WG” motif formed by a limited number of recombination strategies. Modeling of the dominant epitope suggested a flat, featureless structure, but D6M187–195 showed a distinctive structure formed by Lys. The data suggest that common recombination events in prevalent Vβ genes may provide a numerical advantage in the T cell response and that distinct epitope structures may impose more limited options for successful TCR selection. Defining how epitope structure is interpreted to inform T cell function will improve the design of future gene-based vaccines.

It is well established that CD8+ T cells play a pivotal role in the elimination of virus-infected cells (1–4). This is achieved through T cell receptor (TCR)3 recognition of viral peptides in the context of major histocompatibility complex (MHC) class I molecules on the surface of the infected cell. Although many virus-derived peptides are processed and presented, CD8+ T cells respond to a surprisingly small number of viral determinants. Furthermore, a hierarchy can emerge whereby the majority of CD8 T cells respond to one particular viral epitope, a phenomenon labeled immunodominance (5–7). Understanding the basis for immunodominance in the development of primary and memory T cell populations during viral infection will help guide the design of vaccines, particularly gene-based vaccines, with the intended purpose of eliciting broad T cell responses.

Respiratory syncytial virus (RSV) is responsible for a significant global public health burden. RSV infects nearly all infants by 2 years of age, and 0.5–2% of these children require extended hospitalization for severe lower respiratory tract illness (8). RSV infection early in infancy has been associated with recurrent wheezing and asthma later in life (9, 10). Furthermore, prior infection does not incur lifelong immunity. Consequently, half of all infants will be reinfected with RSV by age 2, and immunocompromised and elderly adults remain at risk (11). Thus, the development of an RSV vaccine remains a high priority (12). Although neutralizing antibody can protect infants from severe disease (13, 14), CD8+ T cells can facilitate viral clearance (1) and can modulate the pattern of CD4+ T cell responses to maintain a Th1-dominant response (15, 16) and avoid the patterns of allergic inflammation associated with the RSV vaccine-enhance illness (17, 18). Therefore, defining the rules for eliciting broad, co-dominant CD8+ T cell responses may facilitate RSV vaccine development.

The genetic background of mice has been shown to play a role in the severity of primary RSV infection as well as in the type of response elicited following vaccination and challenge (19, 20). Previously, we identified a pattern of immunodominance hierarchy between two RSV epitopes in a hybrid mouse model. Although the H-2b-restricted M187–195 (NAITNAKII) epitope and the H-2d-restricted M282–90 (SYIGSIINII) epitope are both dominant responses in the respective parent strains, in the F1 hybrid, a distinct and reproducible hierarchy is established with the K4M282–90 dominating the D6M187–195 response (21). Similar phenomena have been described for both influenza and Epstein-Barr virus (EBV) infections (22, 23). Importantly, we have also shown that diminishing the dominant response and improving the subdominant response to make them more co-dominant results in diminished illness (25–27). TCRs are heterodimeric proteins consisting of a β...
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and an α subunit. A repertoire of TCR specificities is established through a process of recombination events in which variable (V), diversity (D), and junctional (J) sequences are selected, modified by trimming or adding nucleotides at the junctions to create additional diversity, and joined with a constant region to form the β subunit. Most of the variability that allows specific recognition of epitopes is found in the complementarity-determining regions (CDR3) of the β subunit. The α subunit provides less variability, but promiscuous pairing of α and β subunits amplifies diversity. The universe of TCRs in a given individual is then tailored through a process of positive and negative selection to result in a collection of T cells capable of recognizing about 10^6 different potential non-self epitopes. Although this process can result in many unique CDR3 sequences, statistical probability and the thymic selection process may result in certain TCR sequences occurring more often than others. Public clonotypes are defined as V-D-J amino acid sequences that are dominant and present in a majority of individuals, and private clonotypes are defined as those rarely observed in multiple individuals. Public sequences can sometimes be achieved by different recombination events resulting in the same nucleotide sequence or different nucleotide sequences encoding the same amino acid sequence, a process termed convergent recombination. For the purposes of this article, a public clonotype is one that is identified in more than one mouse and a private clonotype is one that is identified only in a single mouse. Therefore, the distinction between a public and private clonotype is a relative term because the evaluation of more mice and more sequences may yield additional public clonotypes, although they would be likely to be of low frequency.

In the present study we sought to define the TCR repertoire of RSV-specific CD8+ T cells in the acute response at an early time point after primary infection. T cells were isolated from the lungs of both parent and F1 hybrid mice on day 7 after intranasal RSV infection and sorted with epitope-specific MHC class I tetramers to determine the role of TCR selection in epitope hierarchy. We found that the dominant response has a more public clonotype, restricted largely to a single Vβ subtype, and the subdominant response is much less public, uses a more diverse set of Vβ subtypes, and uses a restricted set of recombination strategies to form a CDR3 motif. Structural modeling suggests that the TCR clonotype may translate structural features of the epitope-MHC complex into functional outcomes seen in T cell responses.

EXPERIMENTAL PROCEDURES

Mice—Pathogen-free BALB/c, C57BL/6, and CB6F1/J female mice were purchased from The Jackson Laboratory. CB6F1/J mice are the F1 generation of a C57BL/6 × BALB/c mating and therefore express both the H-2b and H-2d class I MHC proteins. All animal work was approved by the National Institutes of Health Animal Care and Use Committee. The mice were cared for in accordance with the Guide for the Care and Use of Laboratory Animals as described previously (28). All experiments were performed on age-matched groups. The details of the number of mice and the experiments performed are given in supplemental Table 1.

Virus Infection—The RSV challenge stock was derived from the A2 strain of RSV by sonication of HEP-2 monolayers as described previously (28). Mice were anesthetized intramuscularly with a combination of ketamine (40 μg/ml body weight) and xylazine (6 μg/ml body weight) prior to intranasal inoculation with 10^7 pfu live RSV in 100 μl of 10% Eagle’s minimal essential medium. For peptide scanning experiments, the mice were first immunized with MM2 DNA (50 μg/mice) and challenged with RSV.

Tetramers—Class I MHC tetramers were synthesized by Beckman Coulter. Virus-specific T cells were identified through staining with phycoerythrin-labeled tetrameric complexes of H-2K^d plus the RSV M282-90 peptide or APC-labeled tetrameric complexes of H-2D^b plus the RSV M187-195 peptide.

Cell Isolation and Flow Cytometry Sorting—Mice were sacrificed and lungs harvested at various times post-infection. Spleens were harvested from mice that received vaccination only. Lymphocytes were isolated manually by grinding tissue between the frosted ends of two sterile glass microscope slides in RPMI 1640 medium containing 10% FBS. Lymphocytes were isolated by centrifugation on a cushion of Ficoll-Hypaque at room temperature, washed, resuspended in complete FACS buffer (PBS, 1% FBS, and sodium azide), then stained for surface protein expression of CD8 and either the M2 or M tetramer. Cells were immediately sorted directly into 1.5-ml microtubes containing RNAlater (Ambion) using a modified FACS DIVA (BD Biosciences). 300 to 5000 cells were collected for each experiment. Electronic compensation was conducted with antibody capture beads (BD Biosciences) stained separately with individual antibodies containing the appropriate fluorochromes.

Vβ Antibody Screening—For Vβ antibody screening in naïve mice, splenocytes were harvested from naïve BALB/c, C57BL/6, and CB6F1/J mice and stained with a panel of 15 FITC-labeled Vβ mouse monoclonal antibodies using a mouse Vβ TCR screening panel (BD Biosciences-Pharmin- gen). For Vβ antibody screening in the mice after infection, the lung lymphocytes were harvested and stained with K^dM282-90 and D^bM187-195 tetramers along with FITC-labeled Vβ mouse monoclonal antibodies.

Synthetic Mutant Peptides—Mutant peptides of M2 (SYIGSINNI) and M (NAITNAKII) were obtained from New England Peptide (Gardner, MA). All peptides had a purity of 80% as determined by HPLC. The peptides were dissolved into dimethyl sulfoxide and adjusted to the required concentrations with RPMI culture medium. A total of 16 mutant peptides for M2 and 10 for M were used in the peptide scan experiment.

In Vitro Peptide Stimulation—Spleens from immunized mice were harvested 7 days post-challenge with RSV for in vitro peptide stimulation with mutant peptides. Lymphocytes were isolated as described previously (21) and stimulated with each of the mutant peptide at 2 μg/ml along with the co-stimulatory antibodies (1 μg/ml) to CD28 and CD49d for 5 h at 37 °C. Cells were stimulated with 10 ng/ml phorbol 12-myristate 13-acetate and 1 μM ionomycin as a positive control. After the incubation, cells were fixed and permeabilized ac-
switch-anchored RT-PCR was performed using a 3'-tracted (Oligotex kit, Qiagen), and non-nested, template IFN-γ was expressed as a percentage reduction of the wild-type that recognized the influenza NP366–374 were used as a negative control. Cells were washed after staining and analyzed by flow cytometry on a LSR-II system (BD Biosciences). Flow data were analyzed by FlowJo (version 6.3; Tree Star, San Carlos, CA). Using fluorochrome-conjugated antibodies against CD3, CD8, IFN-γ, and TNF-α (BD Biosciences-Pharmingen) for 20 min at 4 °C. For tetramer analysis, cells were stained with KdM282–90 and DβM187–195 tetramers together with antibodies against CD3 and CD8. Tetramers that recognized the influenza NP366–374 were used as a negative control. Cells were washed after staining and analyzed by flow cytometry on a LSR-II system (BD Biosciences). Flow data were analyzed by FlowJo (version 6.3; Tree Star, San Carlos, CA). Using fluorochrome-conjugated antibodies against CD3, CD8, and IFN-γ, the effect of each amino acid mutation was expressed as a percentage reduction of the wild-type IFN-γ response.

**Bulk Clonotyping**—Sorted cells were lysed, mRNA was extracted (Oligotex kit, Qiagen), and non-nested, template switch-anchored RT-PCR was performed using a 3'- TCRβ constant region primer (5'-TGG CTC AAA CAA GGA GAC CT-3'). Amplified products were ligated into pGEM-T Easy vector (Promega) and cloned by transformation of competent DH5α Escherichia coli. Selected colonies were amplified by PCR using standard M13 primers and then sequenced from an insert-specific primer. A minimum of 50 clones were generated and analyzed per sample. Data analysis was performed using Sequencher, version 4.2 (Gene Codes Corp.). The sequences were analyzed for Vβs by using the IMGT/VQUEST tool from the IMGT® Web site. TCR chain gene usage was determined using the international ImMunoGeneTics (IMGT) information system (29–34). The TCR subtypes of naive lymphocytes were evaluated with FITC-conjugated monoclonal antibodies according to the manufacturer’s directions (mouse Vβ TCR screening panel, BD Biosciences). All TCR usage is designated using the IMGT nomenclature.

**Single Cell Clonotyping**—The tetramer-stained single CD8 T cells were sorted into blank wells of a 96-well plate, and clonotyping was done as described elsewhere (35). Briefly, the Vαs and Vβs were amplified in two rounds of PCR using degenerate primers, and the amplicons were cut from the gel and sequenced after purification to remove the unused nucleotides and primers. An in-house software was used to generate a Vβ summary sheet from which the data were used for further analysis. Web logos of epitope-specific CDR3 sequences were created using WebLogo (University of California, Berkeley) (36).

**RESULTS**

**Epitope-specific Vβ Expression of CD8+ T Cells after Primary RSV Infection in Parent and Hybrid Strains of Mice**—The pre-existing TCR Vβ repertoire of naive BALB/c, C57BL/6, and CB6F1/J mice was screened for TCR usage by staining naive murine splenocytes with a panel of Vβ antibodies conjugated to FITC. We found that in all strains, TCR Vβ13.2/13.3 (these two subtypes are serologically indistinguishable) was present in the greatest frequency (Fig. 1). Next, we determined the pattern of TCR Vβ utilization by RSV-specific CD8+ T cells during primary infection using the antibody panel to label tetramer-positive cells.

Vβ 13.2/13.3 was the dominant response to KdM282–90 recognized by BALB/c parents and CB6F1/J hybrid mice. There were rare Vβ29 responses in BALB/c and Vβ13.1 responses in CB6F1/J hybrids. In C57BL/6 parents and F1 hybrids, the DβM187–195-specific CD8 T cells were primarily Vβ17 followed by Vβ13.2/13.3, with rare responses detected to Vβ 13.1, 14, and 19 in hybrid mice. Other responses were difficult to distinguish from background (Fig. 2 and supplemental Fig. 1).

**Vβ Repertoire of CD8+ T Cells after Primary RSV Infection in Parent and Hybrid Strains of Mice**—Next, we took two approaches to determining the molecular clonotype of the TCR repertoire responding to these two epitopes. First, a bulk...
clonotyping approach was used in which the tetramer-labeled cells were sorted and anchored RT-PCR was done to generate \( V/H \text{9252} \) amplicons. \( V/H \text{9252} \) fragments were cloned into pGEM-T Easy vector and transformed in \( E. \text{coli} \), and the clones were screened by colony PCR and sequenced. The sequences were analyzed to determine the \( V/H \text{9252} \) clonotypes. The data using this approach are shown in supplemental Fig. 2.

Second, we sorted tetramer-specific CD8\(^+\) T cells from the lungs of RSV-infected mice into individual wells for single cell molecular clonotyping. The primary data used for this analysis are from the single cell clonotyping. Comparing the parent strains, the KdM282–90 response in BALB/c used \( V/H \text{9252} \)\(^{13.2}\) almost exclusively, with infrequent (\(<10\%) usage of \( V/H \text{9252} \)\(^{13.1}\) and rare (\(<2\%) usage of \( V/H \text{9252} \)\(^{2, -3, -5, -19, -20, -29, and -31}\) (Fig. 3a). The DbM187–195 responses in C57BL/6 were more equally distributed between \( V/H \) subtypes in this order of frequency: \( V/H \text{9252} \)\(^{17}\), \( V/H \text{9252} \)\(^{13.3}\), \( V/H \text{9252} \)\(^{19}\), and \( V/H \text{9252} \)\(^{20}\). Interestingly, the \( V/H \text{9252} \)\(^{13.1}\) response specific for DbM187–195 was lost, and this was the only \( V/H \) shared with the KdM282–90 response in the parent strains (Fig. 3b). Dominant KdM282–90\(^{*}\) specific responses in both parent and hybrid mice utilized \( V/H \text{B13.2}\), which is the most common \( V/H \) in naive mice from each strain, whereas the DbM187–195 epitope was recognized by a diverse set of less prevalent \( V/H \) subtypes in both parent C57BL/6 or hybrid CB6F1/J mice.

Therefore, it is possible that the more focused KdM282–90\(^{*}\) specific response was able to draw on a larger precursor pool in comparison with the DbM187–195 response. The diversity of \( V/H \) usage in the DbM187–195 response and the loss of \( V/H \text{B13.1} \) clones suggest that the TCR selection may be more complex and requires more CDR3 adaptation in the subdominant response.

**Epitope-specific CDR3 Profiles in Parent and Hybrid Mice after RSV Infection**—Analysis of the hypervariable CDR3 region of the TCR provided additional insight into the diversity of the responding repertoire. Following primary infection, the parental and hybrid mice exhibit diverse CDR3 usage for both the KdM282–90 and DbM187–195 responses. In hybrid mice, KdM282–90\(^{*}\)-specific cells used almost exclusively \( V/H \text{B13.2} \) and \( V/H \text{B13.1} \) (Fig. 3a). As in the parent strains, the DbM187–195\(^{*}\)-specific repertoire exhibited a more diverse repertoire relative to the dominant KdM282–90 response, which included \( V/H \) types in this order of frequency: \( V/H \text{B17}, V/H \text{B13.3}, V/H \text{B19}, \) and \( V/H \text{B20} \). Interestingly, the \( V/H \text{B13.1} \) response specific for DbM187–195 was lost, and this was the only \( V/H \) shared with the KdM282–90 response in the parent strains (Fig. 3b). Dominant KdM282–90\(^{*}\)-specific responses in both parent and hybrid mice utilized \( V/H \text{B13.2} \), which is the most common \( V/H \) in naive mice from each strain, whereas the DbM187–195 epitope was recognized by a diverse set of less prevalent \( V/H \) subtypes in both parent C57BL/6 or hybrid CB6F1/J mice.
CAGGTEVFF (Fig. 4a), accounting for 21.5% of the total sequences. The DbM187–195 response included four public CDR3s; CASSDWGGYEQYF, CASSDWGQDTQYF, and CASSDWGGAEQYF were found in C57BL/6 parents and CB6F1/J hybrids, and CASSERGYEQYF was found only in parents. These public clonotypes accounted for 13% of the total sequences (Fig. 4b). Therefore, the response to the KdM282–90 epitope is more public than the response to the DbM187–195 epitope, again suggesting that there may be a higher pre-existing pool of TCRs for the dominant epitope from which to initiate the response.

The M282–90 Epitope Elicits a More Public Clonotypic Repertoire Than M187–195—To date, when clonotyping data (including bulk clonotyping shown in supplemental Fig. 2, a and b) from all other cumulative experiments are included, we have identified a total of 14 public CDR3 sequences in the KdM282–90 response; these account for 37.6% of the total KdM282–90 response to primary RSV infection. In the DbM187–195 response, we have identified a total of 10 public CDR3 sequences, which make up 19.1% of the total DβM187–195 tetramer-sorted TCR sequences during primary infection (supplemental Fig. 2).

The M187–195 Epitope-specific Response Includes a Longer CDR3 with a Distinct Motif—The length of the CDR3 sequence varied for both epitope-specific responses, ranging from 7 to 14 amino acids for the KdM282–90 response (mean ± S.D., 10.98 ± 1.65) and from 9 to 16 for the DβM187–195 response (mean ± S.D. = 11.95 ± 1.21) (Fig. 5, a and b, respectively). The DβM187–195-specific CDR3 tends to be longer and has a significantly higher proportion of 13 amino acid long CDR3 sequences. This was supported by the bulk clonotyping data (supplemental Fig. 4).

To determine whether the CDR3 sequences were characterized by a particular motif, they were aligned by adding a “Z” to the middle of the shorter sequences. The KdM282–90 epitope-specific sequences follow the general formula GXGXG(Z)\textsubscript{n}XEQ(Y/F)F, with no evidence of a distinct epitope-specific motif, whereas the DβM187–195 response has a dominant sequence pattern, S(D/E)WGG(Z)\textsubscript{n}XT/EQ(Y/F)F, with an epitope-specific motif, “(D/E)WG” (Fig. 6, a and b, respectively).
respectively). A limited number of recombination mechanisms were employed to form the CDR3 motif from the diverse V/H9252 subtypes making up the DbM187–195 response (Fig. 7). The vast majority of (D/E)WG motif-containing TCRs occurred in the V/H9252 13.3, -17, and -19 responses, and only the V/H9252 13.3 and -17 responses had identifiable public clonotypes (supplemental Fig. 3). Although V/H9252 19 sequences commonly used the (D/E)WG motif, all of the CDR3 sequences were unique between mice. Notably, the V/B13.1 that is lost from the D/M187–195 response in CB6F1/J hybrid mice does not appear to have a recombination mechanism that produces the (D/E)WG motif.

In addition to V(D)J recombination, junctional diversity can occur in the D segment or the “diversity” region. Template-independent nucleotide additions (N additions) between the V and D regions and between the D and J regions, respectively. A limited number of recombination mechanisms were employed to form the CDR3 motif from the diverse V/J subtypes making up the D/M187–195 response (Fig. 7). The vast majority of (D/E)WG motif-containing TCRs occurred in the V/J13.3, -17, and -19 responses, and only the V/J13.3 and -17 responses had identifiable public clonotypes (supplemental Fig. 3). Although V/J19 sequences commonly used the (D/E)WG motif, all of the CDR3 sequences were unique between mice. Notably, the V/B13.1 that is lost from the D/M187–195 response in CB6F1/J hybrid mice does not appear to have a recombination mechanism that produces the (D/E)WG motif.

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in addition to template-dependent palindromic nucleotides (P additions), can contribute to the overall diversity of the CDR3 region. Comparing the diversity region in the KdM282–90 and the DbM187–195 responses, there are more limited options for achieving the CDR3 motif for the DbM187–195 response than for the KdM282–90 response. The tryptophan, encoded by the single codon tgg, came from sequences in the D region that were in-frame because of 3′-nucleotide removal from the V region or 5′-nucleotide removal from the D region. N additions were rarely used to align the reading frame to achieve the tryptophan within the (D/E)WG motif (Fig. 7). The (D/E)WG motif was present in 52% of the total TCRs responding to DbM187–195. Interestingly, only about 32% of the motif-containing CDR3 sequences had N additions between the V and D regions, in contrast to 82% in non-motif-containing DbM187–195 CDR3 sequences and 63% in KdM282–90 sequences; the motif sequences had no P1 additions compared with 16% in non-motif DbM187–195 responses and 4% in KdM282–90 responses (Table 1). This indicates that the recombination options for the most common Vβ TCRs bearing the (D/E)WG motif were limited and more likely to occur successfully without N and P additions between the V and D regions. This was particularly true for the Vβ13.3, -17, and -19 responses, where N and P additions were exceedingly rare between V and D. In contrast, the frequency of N additions between D and J was more typical, with the excep-
tion of Vβ19, which had a high frequency (Table 1) and a large number (Fig. 7) of N additions in the D-J recombination site. This may explain why the Vβ19 sequences were relatively unique and private. In contrast, of the (D/E)WG motif-containing sequences in the Vβ13.3 and Vβ17 responses, 43% were public.

The Vβ17 and Vβ19 responses used diversity region 2*01 almost exclusively to encode the tryptophan; Vβ13.3 used both D2*01 and D1*01, and the CDR3 of the DbM187–195 response did not have a tryptophan in the CDR3 region. This suggests that the (D/E)WG CDR3 motif may have conferred some advantage to the DbM187–195 response, to compete with the KdM282–90 response in hybrid mice, thereby limiting the Vβ13.1 response to only the parent strain. However, there is not a major difference in motif frequency comparing the C57BL/6 parent (45 of 99 (45.45%)) with the CB6F1/J hybrid (40 of 75 (53.33%)), so there may be alternative explanations.

The modifications required between the V and D regions were specific to the Vβ/H9252 subtype in the DbM187–195 response. For Vβ17 and Vβ19 there had to be a net removal of seven nucleotides, which was achieved through N deletions and limited additions. For Vβ13.3 there was always a net removal of six nucleotides to put the W codon in-frame (Fig. 7). In contrast, the modifications of the V-D junction in the KdM282–90 response, which resulted in a functional CDR3, were diverse and included N and P additions and nucleotide removal without any apparent need to maintain a particular reading frame. This may explain the relatively low frequency of N and P addition in the V-D recombination site for the DbM187–195 response. There were rare examples of Vβ4 and Vβ31 being used in the M187–195 Response. In the case of Vβ31 the “DWG” motif was preserved, and in Vβ4 a “DWD” sequence was used and shifted downstream one position.

The Lysine Residue (Lys7) of the M Peptide Is Critical for DbM187–195-specific CD8 T Cell Response—Based on the modeling of KdM282–90 (SYIGSINNI) and DbM187–195 (NAITNAKII) peptides (Fig. 8a), Lys7 of the M peptide presents a featured conformation, whereas the M2 peptide is relatively featureless. Although the overall accessible surface area of both peptides was similar (M187–195 (278 Å²) versus M282–90 (258 Å²)), the Lys7 of DbM187–195 is distinctive; it has the highest calculated accessible surface area for a single amino acid residue (123 Å²), representing 44% of the total calculated accessible area, and produces a more vertical structure (Fig. 8b). Ile6 of KdM282–90 is known to be the key amino acid residue for TCR contact in this epitope and has a relatively large accessible area (97 Å²), but modeling suggests that the surface is relatively flat. To define the relative roles of individual amino acids in the interaction with epitope-specific T cells, we produced a panel of mutant peptides bearing an alanine substitution at every residue for the KdM282–90 (SYIGSINNI) and DbM187–195 (NAITNAKII) peptides. A mutant M peptide with a positively charged arginine (Arg7) substitution for the lysine and additional peptides representing each epitope with multiple alanine substitutions were also included in the panel. The peptides were used to stimulate T cells derived from the lungs of RSV-infected mice. The IFN-γ response was abrogated in T cells stimulated with any M-derived peptide bearing the K7A mutation. In addition, the Ile8 and Thr4 residues also played a role in stimulating the TCR (Fig. 8c). The impact of these residues correlated with the predicted surface accessibility of the peptide (Fig. 8b). The corre-

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**TABLE 1**

| Antigen | Sequence | N1 | P1 | N2 | P2 |
|---------|----------|----|----|----|----|
| Vβ CD3  | N        | N  | N  | N  | N  |
| D/EWG   | 90       | 29 | 30 | 0  | 0  |
| Vβ13.3  | 19       | 3  | 10 | 0  | 0  |
| M       | Vβ17     | 48 | 38 | 2  | 1  |
| Vβ19    | 17       | 5  | 16 | 3  | 0  |
| Vβ28.13.01.31.6 | 6 | 5 | 2 | 0 |
| non-D/EWG | 83 | 68 | 62 | 6 |
| M2      | All      | 187| 116| 62 | 7  |

**FIGURE 7.** Examples of the contribution of the junctional diversity to the DβM187–195-specific CDR3 sequence. Selected sequences from the C57BL/6 parents and CB6F1/J hybrids are displayed to provide examples of how the (D/E)WG motif is created and how N and P additions are distributed among the different Vβ subtypes. For the tryptophan (W) codon (tgg) to be in-frame in Vβ17, there had to be a net removal of seven nucleotides, and for Vβ13.3 and Vβ19, there had to be a net removal of six nucleotides. Note the relatively large number of N additions in the D-J recombination site for Vβ19.
FIGURE 8. Modeling of M and M2 epitopes. a, predictive model of the K^dM282–90 and D^bM187–195 peptide-MHC interaction structure is represented. The Lys7 residue in the D^bM187–195 peptide (NAITNAKII) may be responsible for a more distinctive structure in the D^bM187–195 epitope compared with the predicted structure of the K^dM282–90 epitope. The anchor residues are underlined (Asn5 and Ile9 in D^bM187–195 and Tyr2 and Ile9 in K^dM282–90). b, calculated accessible surface areas for each residue of the K^dM282–90 and D^bM187–195 peptides are shown in Å² units. The Ile6 residue and Lys7 of K^dM282–90 and D^bM187–195 peptides, respectively, are predicted to have the most accessible surface exposure area, indicating the key role played by them in the peptide-MHC-TCR interaction. c, normalized reduction in IFN-γ production relative to wild-type peptide is show for mutated peptides with alanine substituted for the indicated residues of K^dM282–90 and D^bM187–195 peptides. Lymphocytes collected from immunized mice 7 days after RSV challenge were stimulated in vitro by a panel of mutant peptides along with wild-type peptides of K^dM282–90 and D^bM187–195. IFN-γ response was determined by intracellular cytokine staining.
TCR Clonotype Influences Response Hierarchy

CD8 T cells respond to a small fraction of the large number of potential immunogenic peptides encoded by viruses. The responses develop a distinct hierarchy determined by a variety of factors related to antigen presentation and recognition resulting in the phenomenon of immunodominance (7, 40). Epitope hierarchies have been well described in other murine models of virus infection including influenza A and LCMV (lymphocytic choriomeningitis virus) (41–43). Immunodominance has also been reported in human CD8 T cell responses to EBV (44), CMV (45), HIV (46–48), HCV (hepatitis C virus) (49), and herpes simplex virus (50). Understanding the basis of epitope hierarchy will be important in designing vaccines that mediate protection through CD8 T cell responses. Although there may be some advantages to engineering vaccines that induce high frequency cytotoxic T lymphocyte responses against a limited number of dominant epitopes, the generation of greater breadth by inducing co-dominant responses to subdominant epitopes may improve the chances for effective and durable immunity. This is particularly true for viruses that can establish chronic infections, especially those with high mutation rates such as HIV-1 or hepatitis C virus (2, 51, 52). Recent evidence suggests that the response to dominant epitopes may not be the most functional, and case studies in HIV have shown that viral replication can be effectively controlled by cytotoxic T lymphocyte responses directed only toward subdominant epitopes (53). Although CD8 T cells specific for dominant epitopes can sometimes control viral replication, there are other examples where the dominant response have poor effector function and weak antiviral activity (53–55). More importantly, having a response to a dominant epitope creates the risk for immune escape through genetic variation and mutation of key residues within the epitope. In the murine model of RSV, various interventions that diminish the dominant response and make the responses more co-dominant diminish illness and immunopathology (25–27). Therefore, defining the properties of numerically subdominant but highly functional T cells may aid the advancement of vaccine development.

The specificity of a T cell response is determined by the TCR interaction with a peptide-MHC complex. However, T cells specific for a particular MHC-peptide are not clonal. They can be distinguished at the level of sequence diversity in the variable domains of the TCR. Each clonotype is formed by a series of recombination steps between V-, D-, and J-region genes with additional diversity contributed by template-dependent and -independent additions and deletions. In this article, we have defined public TCR as two individuals sharing the same Vβ CDR3 and J regions (type 3), as opposed to type 1 (same Vβ but no similarity in CDR3 or J regions) or type 2 (same Vβ and also sharing a CDR3 motif, which can be anywhere between 1 and 4 amino acids) (56).

TCR clonotype compositions have been implicated in mutational escape in acute SIV and immunodominance of SL8/TL8 epitope in Mamu-A*01+ macaques during acute SIV infection, which is targeted by public clonotypes bearing a Vβ CDR3 codon-degenerate motif. Manipulation of the TCR repertoire by vaccination is reported to influence the dominant characteristics of the SL8/TL8 epitope (57). In contrast, immunodominance of D9NP366+CD8+ T cell responses in the influenza A murine model is independent of TCR composition and diversity (58). The present study is the first to describe the RSV-specific clonotypic repertoire of the CD8 T cell response during primary infection in mice. This work addresses the epitope response hierarchy that is established in RSV-infected F1 H-2d/b hybrid mice. We compared the TCR clonotype of the CD8 T cell response to two RSV-specific epitopes in parent strains of mice in which both responses are dominant with the TCR repertoires in F1 hybrid mice in which the CD8 T cell responses assume a distinct hierarchy. We have described the use of Vβ and CDR3 diversity and how epitope structure is interpreted by the TCR repertoire to establish the functional properties of the T cell response.

The K9M282–90 response, which is numerically dominant in the F1 hybrid mouse, utilizes a highly public repertoire that is almost exclusively Vβ13.2. In contrast, the D9M187–195 response, which is numerically subdominant, has a more private repertoire and a more diverse Vβ usage. Interestingly, the CDR3 regions of the T cell responding to D9M187–195 have a distinct (D/E)WG motif, whereas there was no discernable sequence motif in the K9M282–90 response. The discordance in the public nature of the repertoire to the dominant epitope and the sequence motif association with the subdominant epitope suggest that precursor frequency may have contributed more to the former and structural requirements may have been a stronger determinant of the latter.

In addition to the frequency of the epitope-specific TCRs, the precursor frequency of naive TCR Vβs is believed to play a role in the public TCR selection, and it has been proposed that convergent recombination may be responsible for the observed high frequency of some Vβ sequences (59). In the present study, Vβ13.2 was the most commonly used in the K9M282–90 response, and it is also the most prevalent Vβ in all T cells of the parent BALB/c and C57BL/6 mice and the CB6F1/J hybrid, suggesting that the occurrence of this particular Vβ at high frequency in the naive repertoire at one level may have conferred a selective advantage to the K9M282–90 epitope and contributed to its immunodominance. It is interesting to note that Vβ13.1, which does not have a (D/E)WG motif, was used in the M2-specific response of both parents and hybrids and in the M-specific response of C57BL/6 parents but disappears in the hybrid. The fact that it uses a motifless CDR3 sequence may have contributed to its failure to participate in the D9M187–195 response when competing with the K9M282–90 response.

CDR3 region sequence specificity and CDR3 length are reported to be important for making direct contact with the peptide-MHC complex (60–63) and in antigen recognition.
The DbM187–195-specific CDR3 sequence motif may be represented SL8 in H2-Kbm8. This has been attributed to a higher infection compared with congenic C57BL/6 mice that pre-sented SL8 in H2-Kbm8. The DβM187–195 specific CDR3 sequence motif may be required for structural complementarity to the epitope, as it resides in the MHC molecule. This observation is similar to patterns seen in the response to the DβNP366 and DβPA224 epitopes in a C57BL/6 murine model of influenza A infection (69), where TCR diversity has been attributed in part to the structural characteristics of peptide-MHC class I complex (66, 67, 69, 70). In the murine influenza system it was suggested that the flat and featureless DβNP366 epitope from viral nucle-oprotein selected a less diverse and more public set of TCRs compared with the structurally distinct DβPA224 with a P7- arginine that elicits a more diverse and private set of TCRs (70–72). On the basis of the clonotyping data, peptide scanning experiments, and structural modeling studies, we hypothesize that the M epitope (NAITNAKII), with its prominent lysine at position 7 (Fig. 8), requires a specific configuration of the CDR3 region resulting in the selection of a relatively private clonotypic repertoire and strong CDR3 sequence motif, whereas the less featured M2 epitope (SYIGSINNI) is flexible enough to select a more public clonotypic repertoire.

We speculate that the (D/E)WG motif found in TCRs of the DβM187–195 responses may limit the options for recombining the V-D genes, resulting in its numerical subdomi-nance. It is interesting to note that Trp is encoded by just one codon (tgg) and is derived from the D region; so to keep that codon in-frame and to achieve other elements of the motif there had to be a net loss of six nucleotides in the junction between Vβ19 or Vβ13.3 and D and a net loss of seven nucleo-tides between Vβ17 and D in all of the corresponding DβM187–195-specific TCR sequences. This requirement also seemed to limit the frequency of N and P additions in the V-D junction of TCRs involved in the DβM187–195 response. The recombination and addition and deletion options to make the CDR3s associated with the K7M282–90 response were much more diverse, suggesting another potential mechanism for achieving numerical dominance. Various structural (59, 66) and sequence-based (24, 73) explanations have been proposed for public T cell responses. More recently, Venturi et al. (59) proposed that it requires many V-D-J recombination events to form a particular public CDR3 nucleotide sequence, and in the case of a public CDR3 sequence involving a motif, many nucleotide sequences unite to form similar amino acid se-quences, a process called convergent recombination. In our study, although the response to the DβM187–195 epitope is relatively private, among the (D/E)WG motif-containing T cells, 43% were public, based on the single cell data, and over 75% of the public sequences had the motif. Encoding the (D/E)WG motif is limited by the specific number of N additions and deletions that occur at the junctional region between the V and D segments. Convergent recombination implies that a public CDR3 sequence can be encoded by many potential nucleic acid sequences and is therefore statistically more likely to occur. The preferred formation of a CDR3 motif that con-strains TCR selection by requiring conservation of the V-D recombination site would seem to limit the possibilities for convergent recombination, suggesting that selection pressure may be imposed by conformational requirements of the TCR-peptide-MHC interface. Therefore, even though the Vβ sequences in the DβM187–195-specific response containing the motif were relatively public, there were significant constraints on the recombination events needed to produce those sequences, which may have diminished the likelihood of making a functional TCR.

The biophysical properties of the TCR-peptide-MHC interaction play an important role in determining the function of a T cell population. The TCR repertoire is the instrument through which structural informational from the peptide-MHC complex is interpreted. Because TCR diversity is based on stochastic recombination events, there are two major forces that appear to be driving the epitope hierarchy observed in the acute T cell response in this model system. One is the frequency of recombination events that lead to public clonotypes capable of recognizing a particular peptide-MHC complex, and the other is the unique conformational constraints imposed by the peptide-MHC structure. If an epitope is relatively flat and featureless, the chances are greater that publicly available TCR clonotypes will be able to interact with the epitope to produce a greater precursor frequency. Although this could potentially result in a more rapid response, the strength of binding or functional avidity may not be optimal for an interaction between relatively flat surfaces. In contrast, if an epitope has distinct structural features, there may be a limited number of TCR clonotypes capable of an effective interaction, and it would likely have a lower precursor frequency. Although these properties may be the predominant influence on the epitope response hierarchy during the acute phase of primary infection, it will be of interest in future studies to define how properties such as functional avidity and other T cell properties evolve over time. If TCR clonotypes are selected on the basis of distinct structures, it seems likely that, even if they are numerically subdominant initially, the strength of the interaction and subsequent signaling events may play a greater role in functional hierarchy over time. This will be the focus of future studies.

On a practical level, the characterization of Vβ chain usage of CD8 T cells during RSV infection provides the opportunity for generating TCR transgenic mice as a tool for evaluating the role of RSV-specific CD8 T cells in pathogenesis and immunity. These mice will allow for experiments to further explore the basis for epitope dominance including the contribution of precursor frequency and biophysical properties of the TCR-MHC-pep-tide interaction. Ultimately, these studies will provide insight into optimizing the clonal and functional response against subdominant epitopes in future vaccine designs.
TCR Clonotype Influences Response Hierarchy

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