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*J Immunol* 2011; 186:6319-6328; Prepublished online 2 May 2011;
doi: 10.4049/jimmunol.1000883
http://www.jimmunol.org/content/186/11/6319

Supplementary Material  
http://www.jimmunol.org/content/suppl/2011/05/02/jimmunol.1000883.DC1

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Effect of MHC Class I Diversification on Influenza Epitope-Specific CD8+ T Cell Precursor Frequency and Subsequent Effector Function

E. Bridie Day,* Kim L. Charlton,* Nicole L. La Gruta,* Peter C. Doherty,*† and Stephen J. Turner*

Earlier studies of influenza-specific CD8+ T cell immunodominance hierarchies indicated that expression of the H2Kb MHC class I allele greatly diminishes responses to the H2Db-restricted DpPA224 epitope (acid polymerase, residues 224–233 complexed with H2Db). The results suggested that the presence of H2Kb during thymic differentiation led to the deletion of a prominent Vβ7+ subset of DpPA224-specific TCRs. The more recent definition of DpPA224-specific TCR CDR3β repertoires in H2b mice provides a new baseline for looking again at this possible H2Kb effect on DpPA224-specific TCR selection. We found that immune responses to several H2Db- and H2Kb-restricted influenza epitopes were indeed diminished in H2bk F1 versus homozygous mice. In the case of DpPA224, lower numbers of naive precursors were part of the explanation, though a similar decrease in those specific for the Db NP366 epitope did not affect response magnitude. Changes in precursor frequency were not associated with any major loss of TCR diversity and could not fully account for the diminished DpPA224-specific response. Further functional and phenotypic characterization of influenza-specific CD8+ T cells suggested that the expansion and differentiation of the DpPA224-specific set is impaired in the H2bk F1 environment. Thus, the DpPA224 response in H2bk F1 mice is modulated by factors that affect the generation of naive epitope-specific precursors and the expansion and differentiation of these T cells during infection, rather than clonal deletion of a prominent Vβ7+ subset. Such findings illustrate the difficulties of predicting and defining the effects of MHC class I diversification on epitope-specific responses. The Journal of Immunology, 2011, 186: 6319–6328.

Despite the fact that viruses encode multiple proteins, virus-specific CD8+ CTLs focus on a limited number of peptide + MHC class I (pMHCI) epitopes. The relative magnitudes of Ag-expanded pMHCI-specific CTL populations often sort into reproducible hierarchies ranging from large (immunodominant) to small (subdominant) (1). Although many factors may determine the positioning of a given pMHCI complex within this hierarchy, the first requirement is that the peptide should access the nascent MHC molecule during the course of infection, then bind in a way that is recognized by the available CD8+ TCR repertoire (1, 2). Underlying any repertoire effect is the fact that these same MHC alleles that present viral peptides at the time of infection have earlier played a key role in TCR selection during thymocyte ontogeny. A number of studies have examined the impact of differences in MHC1 haplotype and diversity on the generation of epitope-specific CD8+ T cell responses in mice (3–8) and humans (9–11), providing some evidence that the presence of particular MHC1 alleles can modify responses to peptides presented by other MHC1 molecules. Possible mechanisms to explain this effect include changes in the thymic selection of TCRs specific for an individual epitope (6, 10) and altered epitope presentation during infection (11–13). The latter could reflect modulation of MHC1 cell surface expression depending on the combination of MHC1 alleles expressed (13, 14), or that competition for overlapping peptides during infection has the potential to modify epitope processing or presentation (11, 15). Clearly, such MHC1-related effects on response magnitude are of interest as we seek to understand and manipulate CTL-mediated immunity in MHC-polymorphic human populations.

An accumulating body of evidence suggests that epitope-specific TCR diversity plays a part in determining the quality of virus-specific CTL responses and the outcome of infection. More diverse repertoires have been shown to include TCRs with a range of structures and avidities, allowing a spectrum of response profiles that provide better protection against virus infection (16) and minimize the possibility of mutational escape from CD8+ T cell-mediated immune control (17). This has led to the suggestion that vaccination, particularly to protect against viruses (such as HIV and influenza) that readily generate mutants, should optimally prime memory T cell populations with a high level of TCR diversity. Given the role for MHC alleles in shaping the available TCR repertoire (5, 10, 16, 18), it is important to understand how differences in MHC1 haplotype are likely to impact on the frequency, range, and properties of the epitope-specific TCRs selected by immunization, infection, or both.
Early studies of influenza epitope-specific CD8+ T cell responses in mice indicated that expression of the MHCI allele H2Kb greatly diminishes the magnitude of H2Db-restricted responses (4, 8). Subsequent experiments narrowed this effect down to a particular epitope-specific population, demonstrating that virus-infected B6xC3Hf1 (B6C3Hf1, H2b/k) mice mount significantly smaller responses to the DPα224 epitope (acid polymerase, residues 224–233 complexed with H2Db) compared with C57BL/6 (B6, H2b) mice (3). In contrast, responses to the other immunodominant NP366 epitope (nucleoprotein, residues 366–374 complexed with H2Db) were equivalent between the two mouse strains after primary infection. The diminished CD8+DPα224+ responses in the F1 mice looked to be associated with the deletion of a prominent Vβ7+ subset of DPα224-specific TCRs, supporting the idea that thymic development of naive CD8+DPα224+ precursors in the presence of H2Kb had led to a specific “hole” in this TCR repertoire. This result was recorded at a very early stage of our understanding of the Ag-selected CD8+DPα224+ response, long before we had any capacity to measure naive T cell numbers and accurately compare TCR repertoires. Having much better tools available, we repeated this analysis in B6C3F1 mice, with the intent of achieving a much more rigorous definition of the impact of MHCI diversification and possible cross-tolerance (during thymic differentiation) on the selection and expansion of DPα224-specific CD8+ T cells during influenza virus infection. Although this study confirmed a significant reduction in the magnitude of the DPα224-specific response in H2b/k F1 mice, it further identified reduced responses to the subdominant epitopes KBPI731 (polymerase B1, residues 703–711 complexed with H2Kb), DPβ1F256 (polymerase B1 +1 reading frame, residues 62–70 complexed with H2Db), and KNS214 (nonstructural protein 2, residues 114–121 complexed with H2Kb) in F1 compared with B6 mice at all time points examined. Thus, several responses appear to be diminished as a result of MHCI diversification and cross-tolerance. Furthermore, it now seems that our previous conclusion that the CD8+DPα224+ Vβ7+ subset is missing from the B6C3F1 response may have resulted from the selective deletion of these high-avidity T cells during the process of generating peptide-stimulated CD8+DPα224+ cell lines for analysis of Vβ usage. Although the numbers of naive CD8+DPα224+ precursors were reduced in B6C3F1 compared with B6 mice, this was not associated with major changes in TCR repertoire diversity and could not fully account for the diminished response during infection. Overall, it seems that influenza-specific CD8+ T cell responses in B6C3F1 mice may be impeded by factors that affect both the generation of naive CTL precursors (CTLps) and the expansion and differentiation of these precursors subsequent to infection.

Materials and Methods

**Mice and virus infections**

The C57BL/6 (B6, H2b), C3H/HeJ (C3H, H2a), and B6xC3Hf1 (B6C3Hf1, H2b/k) mice were bred and housed in specific pathogen-free conditions at the Department of Microbiology and Immunology at the University of Melbourne (Parkville, VIC, Australia). For analysis of primary responses to influenza virus, mice were anesthetized with methoxyflurane inhalation and infected intranasally (i.n.) with 10⁶ PFU of the influenza virus A/HKx31 (HKx31, H3N2) in 30 µl PBS. Recall responses were examined in mice that were first primed i.p. with 1.5 × 10⁶ PFU of the influenza virus A/PRA/34 (PR8, H1N1) at least 6 wk before i.n. infection with the HKx31 virus. Memory responses were analyzed 6 wk after i.p. priming with either the wild-type or recombinant (19, 20) PR8 viruses. Virus stocks were amplified in the allantoic cavity of day 10 embryonated chicken eggs, and virus titers were determined by plaque assay as PFU on monolayers of Madin-Darby canine kidney cells (21). All experiments were performed with the approval of The University of Melbourne Animal Ethics Committee.

**Tissue sampling and cell preparation**

Lymphocytes were recovered from the infected airways of mice by bronchoalveolar lavage (BAL) and incubated on plastic for 1 h at 37°C to remove adherent cells. Single-cell suspensions were prepared from spleens by pressing through 70 µm cell strainers. In some instances, spleen cells were treated with Tris-buffered ammonium chloride to lyse RBCs.

**Kinetics of virus infection**

For determination of the kinetics of pulmonary virus infection in B6 and B6C3F1 mice, either naive or PR8-primed mice were infected i.n. with the HKx31 virus. At various intervals postinfection, lungs were removed and homogenized in RPMI 1640 (Invitrogen) containing 100 U/ml streptomycin, 100 U/ml penicillin (both Invitrogen), and 24 µg/ml gentamicin (Stelisafe). Virus present in lung homogenates was quantified by plaque assay as described earlier.

**Tetramer and Ab staining**

Lymphocytes were plated at 0.5–2 × 10⁵ cells and incubated with the NP366, DPα224, KBPI731, DPβ1F256, and KNS214 tetramers (ImmunoID) conjugated to either PE or allophycocyanin for 1 h at room temperature. After washing, cells were stained for CD8α and, in some instances, for CD62L. For analysis of Vβ usage, splenocytes from naive mice or tetramer-stained splenocytes from mice sampled on day 10 after HKx31 infection were stained with anti–CD8a-PerCP/Cy5.5 (53-6.7; BD Pharmingen) and a panel of 14 FITC-conjugated mAbs specific for various Vβ families (BD Pharmingen). To identify Ag-experienced CD8+ T cells, we stained splenocytes with anti–CD8a-allophycocyanin (53-6.7; BioLegend) and anti–CD11a-FITC (2D7; BioLegend). For analysis of cell surface H2Db and H2Kε expression, splenocytes from naive mice were treated to lyse erythrocytes and then incubated in Fc block (spent 2.4G2 superantigen with 0.5% normal mouse serum and 0.5% normal rat serum). Cells were then stained with anti–H2Db-FITC (HK95; BD Pharmingen) or anti–H2Kε-FITC (36-7-5; BioLegend). Flow cytometric analysis was performed on a BD FACS Calibur (BD Biosciences), and the data were analyzed by either CellQuest Pro (BD Immunocytometry Systems) or FlowJo (Tree Star) software.

**Sorting, single-cell PCR, and sequencing**

Single CD8+DPα224/Vβ8.3+ or CD8+DPα224/Vβ7+ cells were sorted on a BD FACS Aria (BD Immunocytometry Systems) into the wells of a 96-well PCR plate. cDNA was synthesized, and Vβ8.3+ or Vβ7+ transcripts were amplified and sequenced as described previously (22, 23). The TCR gene nomenclature used here is according to Arden et al. (24).

**Tetramer enrichment**

Enumeration of influenza epitope-specific CD8+ precursors in naive B6 and B6C3F1 mice was performed using a protocol of magnetic enrichment and flow cytometric detection of tetramer binding cells (25) adapted by La Gruta et al. (26). Single-cell suspensions were prepared from spleens and lymph nodes (axillary, brachial, cervical, inguinal, lumbar, mediastinal, mesenteric, pancreatic, and renal) of naive mice. Cells were stained with PE-labeled DPα224 or DPα234 tetramers in Fc block (spent 2.4G2 superantigen with 0.5% normal mouse serum and 0.5% normal rat serum), then washed in cold sorter buffer (PBS containing 0.5% BSA [Life Technologies] and 2 mM EDTA [Ajax Finechem]) and labeled with anti–PE microbeads (Miltenyi Biotech). After further washes, cells were passed over a magnetized LS column (Miltenyi Biotech) according to the manufacturer’s instructions. Cells bound by the column were eluted and stained with anti–CD11b-FITC, anti–CD11c-FITC, anti–B220-FITC, and anti–Ly6G-FITC using a panel of 14 FITC-conjugated mAbs formed a dump gate to exclude cells that nonspecifically bound to tetramers. DPα224 and DPα234-specific CD8+ naive precursors were identified as DUMP CD3+CD4+CD8+ tetramer- cells, with mostly a CD62L+ phenotype.

**Stimulation of CD8+ T cells and intracellular cytokine staining**

Lymphocytes were plated at 0.5–2 × 10⁶ cells and incubated for 5 h either in the presence of plate-bound anti–CD3e (145-2C11; BD Pharmingen) or 1 µM (or graded concentrations for peptide titration experiments) of NP366-374 (ASNENMETM) (27), PA224-233 (SSLENFRAYV) (28), or influenza A virus nucleoprotein residues 50–57 (NP50–57; SDYGERLL) (29) peptides (Auspep) with 10 U/ml human rIL-2 (Roche) and 1 µg/ml Golgi-
plug (BD Biosciences) (30). Cells were then stained for surface expression of CD8α and intracellular IFN-γ, TNF-α, and IL-2 (30). Background levels of staining were determined using controls incubated in the absence of peptide or anti-CD3ε, and were subtracted from percentages obtained for samples incubated in the presence of peptide or anti-CD3ε.

**Tetramer elution assay**

The TCR avidities of DNP366- and DPAP224-specific populations from B6 and B6C3F1 mice were compared using a tetramer elution assay (30). Splenocytes (0.5–2 × 10⁶ cells) were stained with the DNP366-PE or DPAP224-PE tetramers for 1 h at room temperature to label epitope-specific populations. Cells were washed and then incubated in the presence of 5 mg/ml anti-H2Db (28-14-8; BD Pharmingen) at 37°C. At designated time points, cells were removed onto ice, washed, then stained for CD8α expression and analyzed by flow cytometry for residual tetramer staining.

**Statistical analysis**

All statistical comparisons were performed using an unpaired Student t test.

**Results**

**Reduced H2D^b-, H2K^b-, and H2K^k-restricted responses in H2b^k/x F1 mice**

These experiments extend our earlier IFN-γ intracellular cytokine staining assay comparison (3) of CTL responses after HKx31 influenza A virus infection of naive and “memory” B6C3F1 (H2b^k/x) and B6 (H2b^b) mice by using tetramer staining reagents representing the DNP366, DPAP224, KβPB1993, DPB1F262, and KβNS2114 epitopes. Analysis of CTLs from the spleen and infected airways (BAL) 10 d after primary infection with the HKx31 virus (Fig. 1A–D) confirmed (3) that the numbers of CD8^+ DPAP224 cells were ∼8-fold lower in the F1 spleen and ∼20-fold lower in the F1 BAL (Fig. 1B, 1D). The “minor” KβPB1993, DPB1F262, and KβNS2114 responses were also significantly diminished in the F1 mice, but only by 2- to 3-fold (spleen; Fig. 1B). Thus, of the five H2b-restricted epitopes examined, only the DNP366 response was not significantly reduced in the heterozygotes, although it was repeatedly observed to be slightly lower (Fig. 1A–D). As such, the immunodominance hierarchy (Fig. 1A, 1B) in B6C3F1 mice (DNP366 > DPAP224 = Kβ PB1993 = DPB1F262 = KβNS2114) differs from that for the B6 parent (DNP366 = DPAP224 > KβPB1993 = DPB1F262 = KβNS2114). Secondary responses were analyzed in mice immunized i.p. at least 6 wk previously with the H1N1 PR8 virus, then challenged i.n. (Fig. 1A–D). The “minor” KβPB1993, DPB1F262, and KβNS2114 responses were also significantly diminished in the F1 mice, but only by 2- to 3-fold (spleen; Fig. 1B). Thus, of the five H2b-restricted epitopes examined, only the DNP366 response was not significantly reduced in the heterozygotes, although it was repeatedly observed to be slightly lower (Fig. 1A–D). As such, the immunodominance hierarchy (Fig. 1A, 1B) in B6C3F1 mice (DNP366 > DPAP224 = Kβ PB1993 = DPB1F262 = KβNS2114) differs from that for the B6 parent (DNP366 = DPAP224 > KβPB1993 = DPB1F262 = KβNS2114).

A lack of published data on H2k-restricted epitopes recognized in C3H mice limited our ability to compare the influenza-specific CD8^+ T cell responses in C3H and F1 mice (3, 31). However, to demonstrate that F1 mice generated responses to both H2^− and H2^−-restricted viral peptides, we compared primary (Fig. 2A–D), memory (Fig. 2E, 2F), and recall (Fig. 2G–J) responses to the DNP366, DPAP224, and KNP30 epitopes for the parental and F1 mice using an IFN-γ intracellular cytokine staining (30) assay because we did not have tetramer reagents available for the KNP30 epitope. The percentages of DPAP224- and KNP30-specific CD8^+ cells were significantly reduced after primary and secondary challenge of F1 mice; however, as found previously with the tetramers (Fig. 1G–J), this was apparent only for the secondary response to DNP366 (Fig. 2G–J).

**Pathogenicity of influenza virus infection in parenta and F1 mice**

Because B6C3F1 mice generate a broader CD8^+ T cell response targeted toward a greater number of viral peptides (Figs. 1, 2), we reasoned that F1 mice might control influenza more rapidly than the B6 parents, which may, in turn, lead to reduced epitope-specific CTL responses (32). The kinetics of body weight loss and viral clearance were thus compared after primary or secondary challenge of F1 and inbred parental mice (Fig. 3). F1 mice lost significantly less weight compared with both parent strains after primary infection (Fig. 3A). Profiles of weight loss were more similar between the three strains after secondary infection, though B6 mice lost more weight compared with F1 mice between days 2 and 4 (Fig. 3B). There was a high mortality rate among C3H mice after primary infection (7/8 mice), which was reduced when the mice were primed with the PR8 virus i.p. before secondary challenge i.n. (2/5 mice) (Fig. 3A, 3B). This susceptibility to influenza infection may be influenced by a mutation in the TLR4 gene of C3H mice (33), which could make these mice more susceptible to secondary bacterial infections (34), particularly in the absence of pre-existing immunity to influenza virus (Fig. 3A, 3B).

Neither peak titers nor the kinetics of virus clearance differed after primary infection (Fig. 3C), indicating that the reduced magnitude of epitope-specific CD8^+ T cell responses observed in F1 mice (Figs. 1, 2) is not accounted for by any divergence in viral load. However, after secondary challenge, although maximal lung virus titers were comparable for B6, C3H, and F1 mice (Fig. 3D, day 2), the C3H and F1 mice cleared the virus slightly faster (Fig. 3D, day 8), indicating that there are strain-specific differences in the kinetics viral clearance.

**Similar estimates of the total influenza-specific CD8^+ T cell response in H2b^b, H2b^k, and H2b^k/x F1 mice**

It was possible that the total size of the influenza-specific CD8^+ T cell response differed in B6, C3H, and B6C3F1 mice. With only limited information available on the H2b-restricted influenza epitopes recognized in C3H and F1 mice, it was not feasible to enumerate the total influenza-specific CD8^+ T cell responses in mice via tetramer staining or peptide stimulation and IFN-γ detection. We therefore used short-term (5 h) stimulation with anti-CD3 to estimate the size of the total IFN-γ–producing CD8^+ T cell populations in F1 and parental mice, reasoning that during the acute stage of the CD8^+ T cell response to influenza virus infection, the majority of IFN-γ–producing CD8^+ T cells in the spleen and BAL would be influenza-specific. Indeed, after primary challenge with the HKx31 virus, the proportion of IFN-γ–producing CD8^+ T cells detected in the spleens of B6 mice (18.6 ± 5.7%; Fig. 4A) correlated well with the proportion of total influenza-specific CD8^+ T cells calculated by adding up all the H2b-restricted responses measured by tetramer staining (∼15%; Fig. 1A). However, the proportion of IFN-γ–producing CD8^+ T cells detected in the BAL of B6 mice (73.4 ± 5.5%; Fig. 4C) exceeded the proportion of influenza-specific CD8^+ T cells identified by tetramer staining (∼44.3%; Fig. 1C), which could reflect nonspecific recruitment of memory CD8^+ T cells into the lung airways during infection (35). After primary infection, similar proportions and numbers of IFN-γ–producing CD8^+ T cells were detected in the spleen and BAL of B6, C3H, and B6C3F1 mice (Fig. 4A–D), suggesting that the sizes of the total influenza-specific CD8^+ T cell responses are roughly equivalent for the three strains.

Another strategy to compare the size of the influenza-specific CD8^+ T cell responses in B6, C3H, and B6C3F1 mice is exami-
nation of expression levels of CD11a and CD8α to identify and enumerate Ag-experienced CD8+ T cells (CD11ahiCD8αlow) (7). In naive mice, 11% of total CD8+ T cells in the spleen displayed a CD11ahiCD8αlow Ag-experienced phenotype (Fig. 4E). Primary influenza infection led to a substantial increase in the frequency of CD11ahiCD8αlow CD8+ T cells (approaching 40% of all CD8+ T cells in the spleen), and there were no significant differences in the proportion and number of CD11ahiCD8αlow CD8+ T cells between mouse strains. Thus, according to at least two measures, B6, C3H, and B6C3F1 mice appear to generate similar-sized CD8+ T cell responses to influenza virus infection.

Prevalence of epitope-specific CTLps in naive H2bxk F1 mice

It has been suggested that the number of epitope-specific CTLps can substantially influence response magnitude during infection (5, 25, 26, 36). The recent refinement of enrichment/flow cytometry-based protocols (26) allows the reproducible recovery of epitope-specific CTLps from naive secondary lymphoid tissue. We applied this technique to determine whether differences in the DbPA224+ CTLp frequency for B6 and B6C3F1 mice contributed to the reduced response to this epitope in heterozygous mice. Numbers of DbNP366+ CTLps were also determined, because this was the only epitope-specific response that approached a similar magnitude in F1 and B6 mice after primary infection. Interestingly, the naive DbNP366+ CTLp counts were diminished (Fig. 5) 1.7-fold in the B6C3F1 mice (11.1±5.8/mouse) compared with the B6 (18.5±5.2/mouse) control mice (p<0.04), which perhaps explains the earlier observation that F1 DbNP336+CD8+ numbers tended to be slightly lower after primary infection and priming with the PR8 virus (Figs. 1A–F, 2A–F). The prevalence of the naive DbPA224+ CTLp set was reproducibly 2-fold lower (p<0.01) in B6C3F1 (27.8±9.8/mouse) compared with B6 mice (63.8±27.5/mouse). Even so, it is important to note that, despite a 2-fold reduction, the number of DbPA224+ specific CTLps in the heterozygotes is still significantly greater than the number of DbNP366+ CTLps (p<0.005; Fig. 5). Furthermore, the overall decrease in magnitude for the splenic DbPA224+ specific response in B6C3F1 versus B6 mice was at least 8-fold after primary infection (Fig. 1). Thus, although...
the diminished availability of D^{b}PA_{224}^{+} CTLs in B6C3F1 mice may contribute to a reduced response to this epitope during infection, it appears that additional mechanisms are influencing the extent of Ag-driven clonal expansion.

Characterization of TCRV\beta usage in parental and F1 mice

Differences in MHC I haplotype and genetic background can impact on the selection of immune repertoires and the availability of epitope-specific TCRs (5, 16, 37). To determine whether the reduced generation of CD8^{+}D^{b}PA_{224}^{+} and CD8^{+}D^{b}NP_{366}^{+} cells in B6C3F1 mice was associated with modified thymic TCR repertoire selection and possible deletion of particular TCR clonotypes, we compared the prevalence of mAb-defined TCRV\beta families for all CD8^{+} T cells from naive B6, C3H, and B6C3F1 mice (Fig. 6A). Overall, this global measure of V\beta frequency indicated that CD8^{+} T cells from B6C3F1 and C3H mice were more likely (than B6 mice) to express V\beta2, V\beta6, V\beta8.1/8.2, V\beta8.3, and V\beta14, but less likely to stain for V\beta3, V\beta5.1/5.2, and V\beta12. Most striking was the minimal use of V\beta1/5.2 by C3H and B6C3F1 mice (1.1 \pm 0.1% and 0.4 \pm 0.1%, respectively) relative to the B6 strain (13.6 \pm 0.7%). Thus, the TCR\beta repertoire in C3H and B6C3F1 mice differs in some respects from that selected in the B6 strain, suggesting that either C3H non-MHC background genes or the presence of the H2k haplotype is in some way influencing the emergence of particular V\beta families. However, looking at the results (Fig. 6A) for the V\beta8.3 and V\beta7 families that are used prominently in the B6 response to D^{b}NP_{366} and D^{b}PA_{224}, respectively, there were no dramatic differences in prevalence between the F1 and parent.

Our previous analysis described the absence of V\beta7^{+} cells from in vitro expanded D^{b}PA_{224}-specific B6C3F1 CTL lines, with the conclusion that the lack of response was due to a “hole” in the available B6C3F1 repertoire (3). A concern with using this approach to measure V\beta prevalence is that it relies on stimulation with high doses of peptide that may lead to loss of CTLs with high TCR/pMHC avidity (30). The analysis has thus been revisited using contemporary tetramer and Ab staining to measure V\beta usage by D^{b}NP_{366}- and D^{b}PA_{224}-specific CD8^{+} cells taken directly ex vivo from influenza virus-infected B6 and B6C3F1 mice (Fig. 6B, 6C). In B6 mice, V\beta8.3^{+} TCRs are very prominent (42.3 \pm 37.3%; Fig. 6B) in the D^{b}NP_{366}-specific response (22, 38), whereas V\beta7 predominates (55.1 \pm 8.7%; Fig. 6C) for the D^{b}PA_{224}-specific set (3, 23). These V\beta profiles were essentially unaltered in the B6C3F1 mice, with the CD8^{+}D^{b}NP_{366}-V\beta8.3^{+} and CD8^{+}D^{b}PA_{224}-V\beta7^{+} populations representing the major V\beta families used (65 \pm 17.1% and 62 \pm 0.3%, respectively). Although expression of most subdominant V\beta families was similar for CD8^{+}D^{b}NP_{366}^{+} and CD8^{+}D^{b}PA_{224}^{+} cells from B6 and F1 mice, the prevalence of V\beta4 TCRs was increased among F1 CD8^{+}D^{b}PA_{224}^{+} cells (p < 0.05; Fig. 6C). Thus, at this global level, MHC diversification in B6C3F1 mice has not substantially perturbed
epitope-specific TCRβ repertoires. The previous interpretation that CD8^+DbPA224^+Vβ7^+ cells are deleted in B6C3F1 mice during development thus appears to be an artifact resulting from the use of high-dose peptide stimulation to generate in vitro CTL lines.

**Similar CDR3β repertoires for CD8^+DbNP366^+ and CD8^+DbPA224^+ cells from B6 and B6C3F1 mice**

It was possible that the D^αPA224^+specific Vβ7^+ TCRβ repertoire in the B6C3F1 mice might be altered without any obvious change in the overall profile of Vβ usage. This was addressed by comparing the findings for single-cell CDR3β sequence analysis of CD8^+DbPA224^+Vβ7^+ cells from influenza-infected B6C3F1 mice with the extensive published data for the B6 parent (19, 23, 39). The CDR3β repertoire of CD8^+D^αNP366^+Vβ8.3^+ cells (22), which also establish slightly lower precursor frequencies in B6C3F1 and B6 mice (Fig. 5), was analyzed concurrently. The results are summarized in Table I with the F1 CDR3β sequences listed in Supplemental Tables I, II). Thus, the reduced frequency of D^αPA224^+ CD8^+ cells in B6C3F1 mice does not appear to reflect any impaired selection of Vβ7^+ D^αPA224^+specific TCRs. Similarly, obvious differences in TCRβ usage do not explain the reduced number of D^αNP366^+ CTLps in F1 mice.

**Functional and phenotypic differences in influenza-specific CD8^+ T cells from F1 and B6 mice**

A 2-fold reduction in D^αPA224^+ CTLp frequency appeared insufficient to explain the substantial 8-fold reduction in the B6C3F1 D^αPA224^+ CD8^+ response after infection. Furthermore, we had failed to identify differences in the F1 D^αPA224^+specific TCRβ repertoire that could account for this discrepancy. We thus asked whether some difference in the quality of the F1 D^αPA224^+ CD8^+ cells might correlate with this poor expansion during infection.

Immune CD8^+ cells were recovered from the BAL and spleens of F1 and B6 mice on day 8 postinfection with the HKx31 virus and stimulated with the NP366 or PA224 peptides to see whether there was any difference in the cytokine production profiles. Overall, the numbers of CD8^αNP366^+ and CD8^αPA224^+ cells producing IFN-γ (Fig. 7A, 7B) were equivalent to the counts detected by tetramer staining (data not shown), and the level of cytokine production (measured as mean fluorescence intensity) differed only for the D^αNP366^+specific set from spleen (Fig. 7C, 7D). However, looking within the splenic CD8^+IFN-γ^+ population, a greater proportion of both the D^αNP366^+ and D^αPA224^+ specific CD8^+ cells from B6C3F1 mice produced TNF-α (Fig. 7E, 7F) and at higher mean fluorescence intensity levels than in the B6 parent (Fig. 7G, 7H). These B6/F1 differences in TNF-α profiles were much less evident for the highly activated BAL populations (Fig. 7F, 7H).

The CD62L lymph node homing receptor is typically downregulated on influenza-virus–specific CD8^+ CTL effectors and
reflects the extent of differentiation (40, 41). To determine whether levels of CD62L downmodulation were similar between B6 and F1 mice, we compared CD62L expression on CD8+ cells stained with DNP366 and DP244 tetramers from B6 and B6C3F1 mice on day 8 postinfection (Fig. 7I, 7J). The expression of CD62L on CD8+DP244- cells from the spleen and BAL of B6 and B6C3F1 mice was similar. In contrast, significantly fewer T cells within the smaller CD8+DP244- set from the spleens of B6C3F1 mice had downmodulated cell surface CD62L by day 8 postinfection (Fig. 7I); however, again, this effect was no longer apparent in the more highly stimulated BAL environment (Fig. 7J). Both high TNF-α prevalence (Fig. 7E–H) and the retention of the CD62Lhi phenotype (Fig. 7I) are, in fact, characteristic of T cells that have undergone less cycles of division during the course of the host response (40, 42, A.E. Denton, P.C. Doherty, and S.J. Turner, unpublished observations). Thus, the diminished DP244-specific response may reflect an inability to fully differentiate a mature CTL response.

**Functional avidity versus TCR/pMHC binding avidity**

To determine whether differences in T cell responsiveness to peptide stimulation were involved in the impaired expansion of the F1 CD8+DP244+ set, immune CD8+DP244+ and CD8+DP244- cells were stimulated in vitro with graded concentrations of peptides, using IFN-γ production as a readout (Fig. 8A, 8B). Although identical profiles were found throughout for the B6 and F1 CD8+DP244 sets (Fig. 8A), the CD8+DP244+ T cells from B6C3F1 mice were slightly less sensitive at concentrations <10^{-9} M (Fig. 8B). This variation in “functional avidity” for the CD8+DP244+ T cells (Fig. 8B) was independent of TCR avidity, as the rates of tetramer elution were identical (Fig. 8C, 8D) and the B6 and F1 TCR repertoire profiles look to be broadly comparable (Fig. 6, Table I). Thus, the CD8+DP244+ cells from B6C3F1 mice are slightly less responsive to low concentrations of peptide, an effect that may, again, reflect that they have undergone fewer cycles of clonal expansion (43).

**Reduced levels of MHC class I allele expression may contribute to reduced responses in F1 mice**

A way in which MHC class I diversification in F1 mice may affect T cell priming is through altered levels of MHC class I (and thus peptide) presentation. In fact, cell surface expression of H2Dd and H2Kk was reduced in B6C3F1 mice with respect to parental mice (Fig. 9). However, only the DP244-specific CTL response, and not the DNP366-specific CTL response, is substantially affected in F1 mice relative to B6 mice (Figs. 1A–D, 2A–D). This may reflect that DP244 presentation is more limited after influenza A virus infection (19, 44). If levels of Ag presentation are a key determinant of response magnitude in F1 mice, then increasing levels of DP244 epitope presentation could potentially increase the magnitude of the CD8+ T cell response to this epitope in F1 mice. To test this hypothesis, we infected mice with a recombinant PR8-NA PA virus (19) that contains the PA244 peptide inserted into the neuraminidase stalk of the virus. This, in turn, increases the abundance of the PA244 peptide in the influenza virion and facilitates presentation of DP244 on nonprofessional APCs (19). In

**Table I. Summary of CDR3β sequence analysis for CD8+ DP366+Vβ8.3+ and CD8+ DP244+ Vβ7+ cells from B6C3F1 mice after primary infection**

|                      | CD8+ DP366+Vβ8.3+ | CD8+ DP244+ Vβ7+ |
|----------------------|------------------|-----------------|
| No. of mice analyzed | 5                | 5               |
| No. of TCRβ sequences| 344              | 277             |
| Dominant JBα (mean ± SD) | 282 (61.2 ± 9.7%) | 1S1 (14.0 ± 13.0%), 1S5 (13.6 ± 7.4%), 256 (11.6 ± 8.7%) |
| Modal CDR3β length (mean ± SD) | 9 aa (63.2 ± 8.8%) | 6 aa (43.8 ± 13.8%) |
| Clonotypes/mouse    | 5.6 ± 2.1        | 18.8 ± 2.5      |
| No. of public or repeated clonotypes (mean ± SD) | 3 (83.6 ± 14.9%) | 0 (0.0 ± 0.0%) |

*More than 10% of sequences.

*A total of 7.9 ± 2.5 clonotypes/mouse found in B6 (H2b) mice (22).

*A total of 20.6 ± 3.8 clonotypes/mouse found in B6 (H2b) mice (23).

*Found in at least four of five mice.

**FIGURE 7.** Functional and phenotypic differences in influenza-specific CD8+ T cells from B6 and B6C3F1 mice. Mice were infected with the HKx31 virus, and spleen and BAL populations were recovered on day 8. Cytokine expression was analyzed after in vitro stimulation for 5 h with or without 1 μM NP366 or PA244 peptide. Cells were stained for expression of CD8α, IFN-γ (A–D), and TNF-α (E–H). Data show mean numbers of CD8+IFN-γ+ cells after peptide stimulation of spleen and BAL populations (A, B), the proportion of these cells producing TNF-α (E, F) and the MFI of IFN-γ (C, D) and TNF-α staining (G, H). CD62L expression was characterized on CD8+DP366+ and CD8+DP244+ cells from the spleen and BAL (I, J). Data show mean ± SD for four (B6C3F1) or five (B6) mice. *p < 0.05, **p < 0.01 comparing parent with F1.
immunodominance hierarchy established in the H2^{b} (B6) mice was altered in the H2^{ab} (B6C3F1) heterozygotes because of a defect in the response to the D^{b}PA224 epitope. These data demonstrate a broad effect of H2^{k} expression on the magnitude of both immunodominant and subdominant responses. Importantly, reduced primary influenza-specific CTL responses in F1 mice could not be explained by reduced pulmonary virus titers. Numbers of D^{b}PA224^{+} CLTps were reduced in B6C3F1 mice compared with B6 mice, an observation that could not be explained by deletion of a prominent TCR^{b} subset or noticeable differences in TCR CDR3 repertoire. This was unexpected given previous observations suggesting that CD8^{b}D^{b}PA224^{+} V^{b/7} cells were missing from the B6C3F1 response (3). Immune CD8^{b} NP366^{+} and CD8^{b}D^{b}PA224^{+} cells from B6C3F1 also seemed to be less differentiated, and the CD8^{b}D^{b}PA224^{+} set was slightly less sensitive to stimulation with low concentrations of peptide. Together, these data suggest that the F1 environment provides a lower quality of stimulation to these epitope-specific CD8^{+} T cells during infection, which contributes to functional and proliferative differences in these responses compared with the situation for the B6 parent.

The additional MHC and genetic diversity of F1 mice can impact epitope-specific responses in two key ways: via differences in TCR repertoire selection that impact the availability of epitope-specific CTLps and through altered levels of epitope presentation. Although differences in the naive TCR^{b} repertoire were found for B6 and B6C3F1 mice, and seemed a likely explanation for the decrease in (particularly) D^{b}PA224^{+} CTLp frequency, we were unable to identify defined “holes” in the F1 repertoire that could account for this. Previous observations that the V^{b/7} subset of D^{b}PA224^{+} specific TCRs was missing in F1 mice (3) were most likely an artifact of using peptide-stimulated CTL lines, with consequent deletion or lack of expansion of these “high-avidity” TCR clonotypes in culture (30). It remains possible that, if we were to add the analysis of TCR repertoire, we might find some evidence for deletion of F1 CD8^{b}D^{b}NP366 and CD8^{b}D^{b}PA224 clonotypes. However, apart from the fact that the numbers of naive F1 CTLps are down by up to 2-fold, there is no a priori reason for thinking that this might be the case.

Interestingly, the mAb-defined profile of V^{b} usage of CD8^{+} T cells from naive B6C3F1 mice more closely resembled the V^{b} repertoire selected in the C3H parent, rather than representing a mixture of B6 and C3H parental strains. Both MHC and non-MHC gene products are known to skew V^{b} profiles in different mouse strains (45, 46), and in this case, it seems that the C3H background (or H2^{k} haplotype) is to some extent dominant. It is possible that, as a consequence of these differences in TCR repertoire selection, other epitope-specific responses found in B6 mice may be relatively diminished in the B6C3F1, heterozygotes. Indeed, further study will be needed to determine whether such a mechanism is responsible for the decreased responses to K^{b} PB1703, D^{b}PB1-F262, and K^{c}NS2114 in B6C3F1 mice.

The effect of certain MHC alleles on Ag-specific TCR usage has been shown for mice and humans. The HLA-B8 response to an epitope from the EBNA3 protein of EBV (FLRGRAYGL, FL9) selects CD8^{+} cells expressing a public TCR (LC13) (47) that cross-reacts with the alloantigen HLA-B*4402 (48) and is deleted in HLA-B8+HLA-B*4402+ individuals (10). Even so, EBV-infected HLA-B8^{b}/B*4402^{b} individuals still mount a significant FL9-specific response, but use a different, more diverse spectrum of TCRs compared with HLA-B8^{b}/B*4402^{b} individuals. These TCRs can avoid HLA-B^{b}/B*4402 reactivity by adopting a TCR footprint that is shifted to an area of polymorphism between HLA-B8 and HLA-B*4402 (49). Thus, the TCR repertoire has suf-
cient diversity and versatility to ensure that deletion of prominent Ag-specific TCR clonotypes does not severely compromise the magnitude of the response. In a study of vaccinia virus infection of H2d,b F1 mice and inbred parents (5), altered epitope-specific TCRβ profiles in the heterozygotes were not necessarily associated with diminished immune responses. Furthermore, the conservation of parental epitope-specific TCRβ profiles in the F1 mice did not always result in equivalent response magnitudes. Thus, although expression of different MHC alleles can lead to shifts in intrathymic selection of the TCR repertoire to maintain self-tolerance, this does not necessarily compromise the availability of epitope-specific TCRs or the response magnitude. However, contradicting this, a study of influenza-specific CTL responses in humans demonstrated that certain HLA-A– and HLA-B–restricted responses varied in magnitude depending on the HLA haplotype of the individual (9). Although this highlights the significance of MHC-related effects on epitope-specific responses, it remains to be seen whether these effects are mediated at the level of thymic selection or Ag presentation.

The addition of MHC alleles and background genes in F1 mice requires T cells with a greater range of MHC restriction and peptide specificity to be accommodated without a change in overall CD8+ T cell numbers. The prevalence of epitope-specific CD8+ T cells may thus be reduced in F1 mice because of increased positive selection of other TCRs. Indeed, it has been suggested that lower levels of cell surface MHC expression in heterozygous versus homozygous mice may play a role in reducing the positive selection of some epitope-specific TCRs (50), perhaps through a quantitative reduction in TCR signals (51). Differences in MHC glycoprotein levels for cells from parental and F1 mice are, in part, a consequence of gene dosage (13). Analysis of cell surface H2Dk and H2Kk concentrations on splenocytes from B6, C3H, and B6C3F1 mice showed reduced expression of both of these alleles in the F1 versus either parent. In this case, perhaps the decreased levels of H2Dk result in reduced numbers of positively selected DNP366 CD8+ and DP3A224 CD8+ cells and the observed decrease in naive CTLps.

It has been suggested that naive precursor frequency can provide a good prediction of the immunodominance status of epitope-specific responses after Ag-driven clonal expansion (5, 35, 52). However, this relationship did not obviously determine the magnitude of DNP366- and DP3A224-specific responses after influenza infection of B6 and B6C3F1 mice. In both strains, there are more naive DP3A224+ CTLps compared with DNP366+ CTLps; yet, beyond the first few days, the DP3A224-specific response never dominates after normal influenza virus infection (41). Given the reduced H2Dk expression in F1 mice, it is tempting to speculate that the levels of cell surface DNP366 and DP3A224 presentation may be selectively diminished in the F1, with consequent reduction in the quantity and quality of stimulation delivered to CD8+ T cells. However, any decrease in DNP366 presentation does not appear to substantially affect relative response magnitudes in B6C3F1 versus B6 mice. This may reflect differences in the presentation profiles of DNP366 and DP3A224. It has been demonstrated that during infection, a broader array of cell types are capable of presenting the DNP366 epitope compared with the DP3A224 epitope (20, 44). Thus, even though expression of H2Dk is reduced in F1 compared with B6 mice, levels of DNP366 presentation will likely be greater than DP3A224, and therefore sufficient, to prime a response in F1 mice similar in magnitude to that observed in B6 mice after primary infection. In contrast, the generally lower abundance of the PA protein, coupled with reduced H2Dk levels in B6C3F1 mice, may result in DP3A224 presentation that is decreased for both duration and concentration, with a consequent decline in clonal expansion and magnitude for responding CTL populations. In this way, a 2-fold lower precursor frequency could be extended to an 8-fold difference in numbers of splenic DP3A224 CD8+ T cells after primary infection. Interestingly, others have also found that relatively small differences in epitope-specific CTLp frequency between F1 mice and inbred parents can result in much larger differences in effector CTL numbers after infection or peptide vaccination (5). Finally, we cannot rule out the possibility that differences in DP3A224-specific response magnitude could reflect differences in Ag processing and presentation between B6C3F1 and B6 mice. It has been reported that presentation of the PA224 epitope is exquisitely sensitive to expression of specific immunoproteasome subunits (53). Thus, it remains a possibility that there is limited upregulation of immunoproteosome expression within the B6C3F1 mice that impacts PA224 processing and presentation. This intriguing possibility remains a possible avenue of future study.

Consistent with decreased proliferation and maturation of the response (40, 42) and A.E. Denton, P.C. Doherty, and S.J. Turner, unpublished observations], a greater proportion of CD8+DP3A224+ cells from B6C3F1 mice produced TNF-α (and in higher amounts) and more were CD62L(43). Furthermore, despite equivalent structural TCR avidity, the B6C3F1 CD8+DP3A224+ population was slightly less sensitive to stimulation with peptide. Taken together, these data suggest that responses to DP3A224 are poorly elicited in the B6C3F1 environment and fail to reach their full potential for expansion and function. Indeed, several of the very early studies looking into the effect of MHC haplotype on virus-specific CTL responses suggested that the presence of an H2Kk environment during infection and not lack of precursors was responsible for limiting the potential of H2Dk-restricted, influenza-specific responses (4, 8).

As shown in this article, MHC diversification can substantially affect the selection and expansion of many epitope-specific CD8+ T populations. The explanation of these effects is clearly not as simple as previously thought (3) and involves multiple factors that affect the generation of naive precursors, as well as the proliferation and activation of these precursors during infection. Although this certainly presents challenges for the design of T cell-directed vaccines for use in MHC-diverse human populations, these studies have provided key insights into the complexity of parameters that shape CTL response magnitude where MHC diversification is apparent.

Acknowledgments

We thank Drs. A.E. Denton, M.R. Olson, K. Kedzierska, J. Stambas, and B.E. Russ for helpful discussions.

Disclosures

The authors have no financial conflicts of interest.

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