

# Complete modification of TCR specificity and repertoire selection does not perturb a CD8<sup>+</sup> T cell immunodominance hierarchy

Katherine Kedzierska<sup>a,1</sup>, Carole Guillonneau<sup>a</sup>, Stephanie Gras<sup>b</sup>, Lauren A. Hatton<sup>a</sup>, Richard Webby<sup>c</sup>, Anthony W. Purcell<sup>d</sup>, Jamie Rossjohn<sup>b</sup>, Peter C. Doherty<sup>a,e,1</sup>, and Stephen J. Turner<sup>a</sup>

<sup>a</sup>Department of Microbiology and Immunology, and <sup>d</sup>Department of Biochemistry and Molecular Biology, The Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Parkville 3010, Melbourne, Australia; <sup>b</sup>Protein Crystallography Unit, and Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria 3800, Australia; and Departments of <sup>c</sup>Infectious Diseases and <sup>e</sup>Immunology, St. Jude Children's Research Hospital, Memphis, TN 38105

Contributed by Peter C. Doherty, October 15, 2008 (sent for review July 22, 2008)

**Understanding T cell immunodominance hierarchies is fundamental to the development of cellular-based vaccines and immunotherapy. A combination of influenza virus infection in C57BL/6J mice and reverse genetics is used here to dissect the role of T cell antigen receptor (TCR) repertoire in the immunodominant D<sup>b</sup>NP<sub>366</sub>CD8<sup>+</sup> T cell response. Infection with an engineered virus (NPM6A) containing a single alanine (A) mutation at the critical p6 NP<sub>366-374</sub> residue induced a noncross-reactive CD8<sup>+</sup> T cell response characterized by a novel, narrower TCR repertoire per individual mouse that was nonetheless equivalent in magnitude to that generated after WT virus challenge. Although of lower overall avidity, the levels of both cytotoxic T lymphocyte activity and cytokine production were comparable with those seen for the native response. Importantly, the overdominance profile characteristic of secondary D<sup>b</sup>NP<sub>366</sub>-specific clonal expansions was retained for the NPM6A mutant. The primary determinants of immunodominance in this endogenous, non-TCR-transgenic model of viral immunity are thus independent of TCR repertoire composition and diversity. These findings both highlight the importance of effective antigen dose for T cell vaccination and/or immunotherapy and demonstrate the feasibility of priming the memory T cell compartment with engineered viruses to protect against commonly selected mutants viral (or tumor) escape mutants.**

avidity | viral escape | pMHC structure | TCR diversity | reverse genetics

Virus-specific CD8<sup>+</sup> T cells play an essential role in limiting infectious process by killing virus-infected cells and/or producing proinflammatory cytokines (1). Because this CD8<sup>+</sup> T cell-mediated immunity tends to be directed at 1 or 2 immunodominant epitopes and a number of subdominant determinants (2), we need to identify the major factors controlling immunodominance if we are to develop optimized T cell vaccines and immunotherapy protocols. Influenza A virus infection of C57BL/6J (B6, H2<sup>b</sup>) mice provides a well-characterized “natural” [non-T cell receptor (TCR)-transgenic] system for dissecting CD8<sup>+</sup> T cell immunodominance hierarchies. Although there are at least 6 epitopes distributed between H2D<sup>b</sup> and H2K<sup>b</sup> (3), the most prominent CD8<sup>+</sup> sets that emerge after an initial encounter with an influenza A virus are specific for H2D<sup>b</sup>-bound peptides from the viral nucleoprotein (D<sup>b</sup>NP<sub>366-374</sub>) and acid polymerase (D<sup>b</sup>PA<sub>224-233</sub>) proteins (4). These D<sup>b</sup>NP<sub>366</sub><sup>+</sup>CD8<sup>+</sup> and D<sup>b</sup>PA<sub>224</sub><sup>+</sup>CD8<sup>+</sup> populations reach essentially comparable sizes after primary infection, although the D<sup>b</sup>PA<sub>224</sub><sup>+</sup>CD8<sup>+</sup> set peaks 1–2 days earlier (5), reflecting a higher naïve T cell precursor frequency (6, 7). After secondary challenge, the D<sup>b</sup>NP<sub>366</sub><sup>+</sup>CD8<sup>+</sup> T cells are massively overdominant, constituting up to 80% of the total virus-specific CD8<sup>+</sup> response (8). This emphasis on D<sup>b</sup>NP<sub>366</sub> in the recall response has been attributed to differences in the spectrum of antigen-presenting cell involvement (9), protein/peptide abundance, and T cell precursor frequency (7).

TCR repertoires selected by D<sup>b</sup>NP<sub>366</sub> and D<sup>b</sup>PA<sub>224</sub> differ in both extent and character. Analysis of TCR CDR3 $\beta$  sequence variability and clonal prevalence shows that D<sup>b</sup>PA<sub>224</sub> recruits a predominantly “private” (specific for individual mice) and diverse range of TCR $\beta$  sequences (10), whereas CD8<sup>+</sup> T cell recognition of D<sup>b</sup>NP<sub>366</sub> is mediated via a narrower, “public” (conserved between individuals) TCR repertoire (11). Could selection of optimal public clones by D<sup>b</sup>NP<sub>366</sub> but not D<sup>b</sup>PA<sub>224</sub> explain the dramatic divergence in CD8<sup>+</sup> T cell response magnitude, especially after secondary challenge? To date, the role of TCR fine specificity and particular TCR clones in establishing immunodominance hierarchies remains unclear. The present analysis asks whether the limited, public TCR repertoire characteristic of the D<sup>b</sup>NP<sub>366</sub><sup>+</sup>CD8<sup>+</sup> T cell response is a primary determinant of immunodominance.

## Results

**Residues Critical for TCR Recognition by D<sup>b</sup>NP<sub>366</sub><sup>+</sup>CD8<sup>+</sup> T Cells.** To determine critical residues for recognition of the NP<sub>366</sub> peptide by H2D<sup>b</sup>-restricted CD8<sup>+</sup> T cells, single amino acid mutations were made at different positions within the viral NP<sub>366</sub> peptide, excluding the anchor amino acid (p5, p9). These included A substitutions (S2A, N3A, E4A, M6A, E7A, T8A), conserved (E4D, M6Q, E7D, T8S), and reverse charge (E4K, E7K) mutations. Mutant NP<sub>366</sub> peptides were used to probe CD8<sup>+</sup> T cells by intracellular cytokine secretion (ICS), a <sup>51</sup>Cr-release assay and stimulation of D<sup>b</sup>NP<sub>366</sub><sup>+</sup>LacZ-inducible T cell hybridomas. The recognition profiles of polyclonal D<sup>b</sup>NP<sub>366</sub><sup>+</sup>CD8<sup>+</sup> T cells obtained from mice infected with the WT virus were variably modified by different amino acid substitutions (Fig. 1A and B). p6M appeared to be critical for TCR recognition, as stimulation with 2 different amino acid substitutions (M6A, M6Q) resulted in a complete loss of peptide-induced IFN- $\gamma$  production (Fig. 1A) and <sup>51</sup>Cr-mediated lysis (Fig. 1B). This finding is in accord with data showing that the emergence of viral escape mutants in mice transgenic for a D<sup>b</sup>NP<sub>366</sub><sup>+</sup> TCR resulted from a p6M mutation (12). The p4E position was also important, while substitutions at p3, p7, and p8 led to a partial loss of TCR recognition (Fig. 1A). Stimulating 3 distinct D<sup>b</sup>NP<sub>366</sub><sup>+</sup> LacZ-

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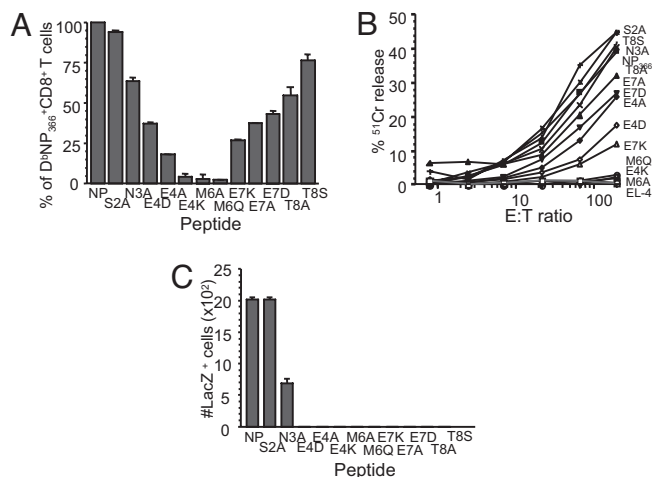
The authors declare no conflict of interest.

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 3CPL).

<sup>1</sup>To whom correspondence may be addressed. E-mail: kkedz@unimelb.edu.au or pcd@unimelb.edu.au.

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**Fig. 1.** Residues important for TCR recognition by D<sup>b</sup>NP<sub>366</sub><sup>+</sup> CD8<sup>+</sup> T cells. (A and B) Splenocytes obtained from PR (H1N1)-primed mice secondarily-infected with the WT HK (H3N2) influenza virus were stimulated with either the WT NP<sub>366-374</sub> or a panel of mutant peptides with single amino acid substitutions. The extent of TCR recognition was assessed by IFN- $\gamma$  production in the ICS assay (A) and <sup>51</sup>Cr cytotoxicity after incubation with target EL-4 cells pulsed with 1  $\mu$ M peptides and 750  $\mu$ Ci <sup>51</sup>Cr (B). (C) The response profiles of monoclonal D<sup>b</sup>NP<sub>366</sub><sup>+</sup> LacZ-inducible hybridomas expressing the public SGGGNTGQL CDR3 $\beta$  (11) to WT NP<sub>366</sub> and NP-mutant peptides are shown.

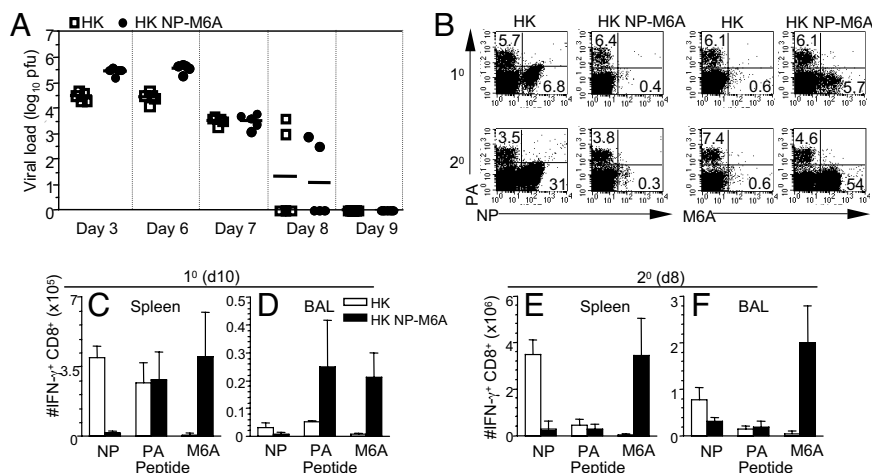
inducible hybridomas expressing 1 of the D<sup>b</sup>NP<sub>366</sub><sup>+</sup> public TCR $\beta$  sequences, SGGGNTGQL (11), with NP-mutant peptides led to different results. Monoclonal, public-TCR, D<sup>b</sup>NP<sub>366</sub><sup>+</sup> T cells responded optimally to only WT NP<sub>366</sub> and NPS2A peptides and partially to the NPN3A peptide (Fig. 1C). Thus, although polyclonal D<sup>b</sup>NP<sub>366</sub>CD8<sup>+</sup> T cells can recognize a spectrum of mutant NP<sub>366</sub> peptides, the public D<sup>b</sup>NP<sub>366</sub><sup>+</sup> hybridomas do not tolerate mutations at p4, p6, p7, and p8, supporting the idea that limiting the diversity of antigen-specific TCRs has the potential to facilitate the emergence of mutant viruses (13).

**Characteristics of Infection and CD8<sup>+</sup> T Cell Response Magnitude for the NPM6A Virus.** Having shown that p6M is critical for TCR recognition of D<sup>b</sup>NP<sub>366</sub>, we expressed the M6A mutation within

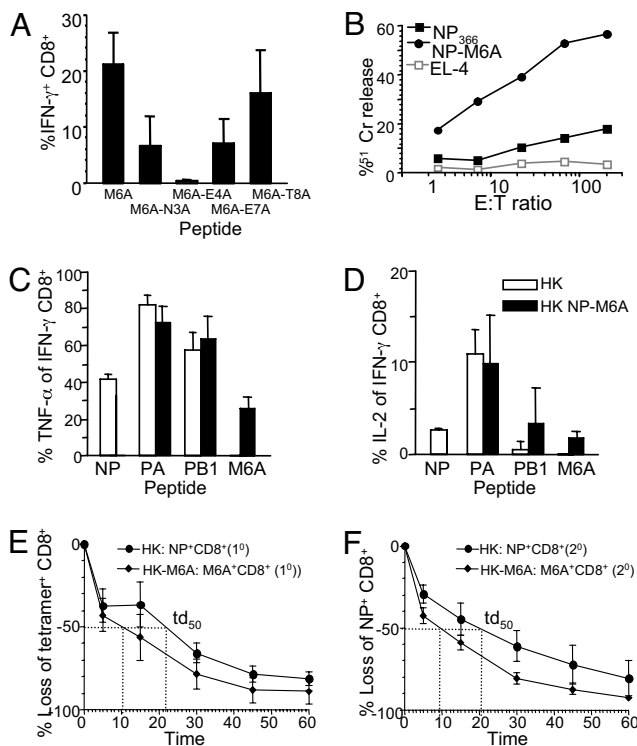
the viral NP<sub>366</sub> sequence to determine whether it would abrogate the WT D<sup>b</sup>NP<sub>366</sub><sup>+</sup>CD8<sup>+</sup> T cell response. What TCR repertoire might the new D<sup>b</sup>NPM6A epitope select after infection? Would the utilization of a novel TCR repertoire for D<sup>b</sup>NPM6A modify quantitative and qualitative aspects of the influenza NP-specific CD8<sup>+</sup> T cell response? A single A substitution at p6 was engineered to create an M6A mutation within the viral NP<sub>366</sub> peptide. Two serologically distinct viruses with the NPM6A change were generated by using the standard HKx31 (HK-NPM6A) and PR8 (PR-NPM6A) strains, to facilitate prime/boost experiments in the absence of cross-reactive antibodies.

The WT and mutant NPM6A viruses replicated to equivalent titers in B6 mice (Fig. 2A), suggesting no diminution in viral fitness (and thus NP amount and kinetics of production) as a consequence of the single amino acid change in this virus structural protein. In fact, the lung titers for HK-NPM6A were significantly ( $P > 0.05$ ) higher on days 3 and 6, although both viruses were cleared with equivalent kinetics. This finding indicates that the level and duration of viral protein production is broadly comparable for the NPM6A and WT NP proteins. Furthermore, because NP<sub>366</sub> and NPM6A peptides bind to the H-2D<sup>b</sup> MHC glycoprotein with equivalent affinity (14) and the thermostability [supporting information (SI) Fig. S1] of H2D<sup>b</sup>-NPM6A midpoint of thermal denaturation ( $T_m$ ) of  $63.3 \pm 0.7^\circ\text{C}$  is higher than that for the native H2D<sup>b</sup>-NP<sub>366</sub> ( $T_m$  of  $51.8 \pm 0.7^\circ\text{C}$ ), there is no reason to think that the mutant NPM6A peptide is any less “fit” than the WT NP<sub>366</sub> when it comes to the generation of an immunogenic epitope.

Primary and recall CD8<sup>+</sup> T cell responses were generated by infecting naïve and PR i.p.-immunized B6 mice intranasally (i.n.) with the HK variants. Spleen (Fig. 2B, C, and E) and bronchoalveolar lavage (BAL) (Fig. 2D and F) CD8<sup>+</sup> T cells were analyzed on day 10 (1<sup>o</sup>) or day 8 (2<sup>o</sup>) by staining with the D<sup>b</sup>NP<sub>366</sub> and D<sup>b</sup>NPM6A tetramers (Fig. 2B) or stimulation with peptide (Fig. 2C–F). The viruses carrying the NPM6A mutation induced the recruitment and expansion of naïve (Fig. 2B, C, and E) and memory (Fig. 2B, E, and F) D<sup>b</sup>NPM6A-specific CD8<sup>+</sup> T cells at levels equivalent to the WT D<sup>b</sup>NP<sub>366</sub><sup>+</sup>CD8<sup>+</sup> set (8) (Fig. 2B–F). It seems that the M-to-A substitution at p6 in the NP<sub>366</sub> peptide created a novel peptide/MHC I complex (pMHCI) that is antigenically distinct from, but as immunogenic as, the WT epitope. Although the response magnitudes were similar in spleen (a good reflection of immunodominance), slightly larger



**Fig. 2.** Virus clearance and CD8<sup>+</sup> T cell profiles after infection with the WT HK and mutant HK-NPM6A viruses. Mice were infected with WT HK or the mutant HK-NPM6A virus. (A) Lungs were sampled at days 3, 6, 7, 8, and 9 after infection and titrated in a plaque assay. The results are log<sub>10</sub> pfu per lung ( $n = 5$ ). (B) CD8<sup>+</sup> T cell responses were measured after 1<sup>o</sup> (day 10) or 2<sup>o</sup> (day 8) i.n. challenge of naïve or PR- or PR-NPM6A-primed mice with the homologous HK WT or HK-NPM6A virus. Cells were stained with the D<sup>b</sup>NP<sub>366</sub>-APC, D<sup>b</sup>NP-M6A-APC, or D<sup>b</sup>PA-PE tetramers. (C–F) The response magnitude was assessed for spleen (C and E) and BAL (D and F) sets by ICS. Datasets are mean  $\pm$  SD for  $n = 5$ .



**Fig. 3.** Functional quality of the emerging M6A<sup>+</sup>CD8<sup>+</sup> sets. D<sup>b</sup>NPM6A<sup>+</sup>CD8<sup>+</sup> T cells generated after challenge with the HK-NPM6A virus were assessed for important residues in TCR recognition (A), <sup>51</sup>Cr-mediated killing (B), production of IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 (C and D), and TCR avidity for the pMHC complex (E and F). (A) Cells obtained from mice infected with NPM6A viruses were incubated with the NPM6A (or mutant peptides with further A mutations) to stimulate IFN- $\gamma$  production. (B) Cells of mice secondarily challenged with the HK-NPM6A virus were cultured for 4 h. <sup>51</sup>Cr-labeled EL-4 cells were pulsed with 1  $\mu$ M of NP<sub>366</sub> or NPM6A peptides. (C and D) Data ( $n = 5$ , mean  $\pm$  SD) for hierarchical production of IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 after 1<sup>o</sup> or 2<sup>o</sup> infection with either WT HK or mutant HK-NPM6A viruses. (E and F) Tetramer dissociation as a measure of TCR avidity. Cells were stained with the D<sup>b</sup>NP<sub>336</sub> or D<sup>b</sup>NPM6A tetramers and incubated with a mAb to H2D<sup>b</sup> to prevent rebinding of dissociated tetramer. The progressive diminution in tetramer staining was measured. Td50 defines time to 50% tetramer loss. Data represent mean  $\pm$  SD,  $n = 5$ .

NPM6A<sup>+</sup>CD8<sup>+</sup> T cell numbers were recovered from the site of infection (BAL), perhaps reflecting the elevated antigen load on days 3 and 6 after viral exposure (Fig. 2A). Furthermore, the characteristic WT CD8<sup>+</sup> T cell immunodominance hierarchies were maintained after exposure to viruses carrying the NPM6A mutation. As found for WT infections that induce expansion of the D<sup>b</sup>NP<sub>366</sub>CD8<sup>+</sup> set, the primary responses to D<sup>b</sup>NPM6A and D<sup>b</sup>PA<sub>224</sub> in lymphoid tissue look to be essentially comparable in size (Fig. 2 C and E). However, the secondary response to D<sup>b</sup>NPM6A showed the massive overdominance (relative to D<sup>b</sup>PA<sub>224</sub>) found invariably for the WT D<sup>b</sup>NP<sub>366</sub><sup>+</sup>CD8<sup>+</sup> set. Thus, although CD8<sup>+</sup> T cells induced by the D<sup>b</sup>NP<sub>366</sub> and D<sup>b</sup>M6A epitopes show little evidence of cross-reactivity, the responses to these 2 epitopes are similar, at least in the numerical sense of immunodominance.

**Consequences for CD8<sup>+</sup> T Cell Recognition and Function.** Analysis of response profiles with mutated peptides (Fig. 1A) was repeated (Fig. 3A) for the responding NPM6A<sup>+</sup>CD8<sup>+</sup> T cells. Starting with NPM6A, we made a panel of single A substitutions (M6A-N3A, M6A-E4A, M6A-E7A, M6A-T8A) to identify the residues critical for TCR recognition. Stimulation of T cells recovered directly from mice infected with the HK-NPM6A virus estab-

lished that polyclonal NPM6A<sup>+</sup>CD8<sup>+</sup> T cells respond equivalently to the M6A and M6A-T8A peptides, respond partially to M6A-N3A and M6A-E7A, and cannot recognize the M6A-E4A mutation (Fig. 3A). This finding shows that the residue critical for TCR recognition by D<sup>b</sup>NPM6A<sup>+</sup>CD8<sup>+</sup> T cells is now shifted to p4 E.

The inference from the in vivo response experiments (Fig. 2) and the mutational analysis (Fig. 3A) that D<sup>b</sup>NP<sub>366</sub> and D<sup>b</sup>NPM6A are distinct epitopes was further supported by the lack of cross-reactivity for cytotoxic T lymphocyte (CTL) populations when tested in a <sup>51</sup>Cr release assay (Fig. 3B). The capacity to produce multiple cytokines (4) simultaneously (IFN- $\gamma$ , TNF- $\alpha$ , and IL-2) showed that frequencies of double producers (IFN- $\gamma$ /TNF- $\alpha$ ) and triple producers (IFN- $\gamma$ /TNF- $\alpha$ /IL-2) within the D<sup>b</sup>NPM6A<sup>+</sup>CD8<sup>+</sup> set were equivalent to those found for the D<sup>b</sup>NP<sub>366</sub><sup>+</sup>CD8<sup>+</sup> populations but were significantly lower than the values for D<sup>b</sup>PA<sub>224</sub><sup>+</sup>CD8<sup>+</sup> T cells stimulated by the WT or NPM6A influenza viruses (Fig. 3 C and D). This finding, together with the CTL results (Fig. 3B), suggests that the D<sup>b</sup>NPM6A<sup>+</sup>CD8<sup>+</sup> and D<sup>b</sup>NP<sub>366</sub><sup>+</sup>CD8<sup>+</sup> T cells are functionally comparable, although they differ in specificity. Binding avidities were compared by the tetramer dissociation rate (measuring the “off” rate component of avidity) (4) for D<sup>b</sup>NPM6A<sup>+</sup>CD8<sup>+</sup> and D<sup>b</sup>NP<sub>366</sub><sup>+</sup>CD8<sup>+</sup> populations. The tetramer dissociation curves were compared for D<sup>b</sup>NPM6A<sup>+</sup>CD8<sup>+</sup> and D<sup>b</sup>NP<sub>366</sub><sup>+</sup>CD8<sup>+</sup> TCRs after primary (Fig. 3E) and secondary (Fig. 3F) infection. The td50 value (the time to 50% tetramer loss) was much shorter for the D<sup>b</sup>NPM6A<sup>+</sup>CD8<sup>+</sup> ( $t = 10.1$  min and  $t = 10.0$  min for 1<sup>o</sup> and 2<sup>o</sup> responses) than for the WT D<sup>b</sup>NP<sub>366</sub><sup>+</sup>CD8<sup>+</sup> T cells ( $t = 21.8$  min and  $t = 22.0$  min] for 1<sup>o</sup> and 2<sup>o</sup>, respectively), indicating that, on a population basis, the D<sup>b</sup>NPM6A epitope selects TCRs that bind less strongly. Furthermore, TCR/pMHC avidity was found to be lower for D<sup>b</sup>NPM6A by 2 other measures, tetramer dilution and CD8 $\beta$  dependence assays (Fig. S2). In addition, there was no evidence of pMHC–TCR avidity maturation from the primary to the secondary response.

**D<sup>b</sup>NPM6A Selects a Distinct and Less Diverse TCRV $\beta$  Repertoire.** To show that the novel D<sup>b</sup>NPM6A epitope selects an array of TCRs different to the native D<sup>b</sup>NP<sub>336</sub>, we defined the D<sup>b</sup>NPM6A<sup>+</sup>CD8<sup>+</sup> repertoire at the clonal level. First, the D<sup>b</sup>NPM6A<sup>+</sup>CD8<sup>+</sup> T cell sets were analyzed for V $\beta$  usage. The strong V $\beta$ 8.3 bias characteristic of D<sup>b</sup>NP<sub>366</sub><sup>+</sup>CD8<sup>+</sup> T cells was not apparent for the D<sup>b</sup>NPM6A<sup>+</sup>CD8<sup>+</sup> sets (Fig. S3), which use a broader and less consistent spectrum of TCRV $\beta$  elements. Unlike D<sup>b</sup>NP<sub>366</sub><sup>+</sup>CD8<sup>+</sup> T cells, D<sup>b</sup>NPM6A did not select consistent TCRV $\beta$  profiles. The responses were individualized, with an average (per mouse) of 2.8 (1<sup>o</sup>) and 2.2 (2<sup>o</sup>) prominent TCRV $\beta$  elements after HK-NPM6A infection (Tables S1), and S2). Clonal analysis of 996 sequences from specific V $\beta$  segments showed that, although the D<sup>b</sup>NPM6A<sup>+</sup>CD8<sup>+</sup> T cells used multiple V $\beta$  regions, the extent of TCR repertoire selection within an individual TCRV $\beta$ <sup>+</sup> set was quite restricted (Table S1 and S2). An average of 3.3 and 2.4 clonotypes were identified for each D<sup>b</sup>NPM6A<sup>+</sup>CD8<sup>+</sup> TCRV $\beta$  after 1<sup>o</sup> or 2<sup>o</sup> challenge (Table S2), which is lower than the average of 7.9 clonotypes characteristic of the D<sup>b</sup>NP<sub>366</sub><sup>+</sup>V $\beta$ 8.3<sup>+</sup>CD8<sup>+</sup> T cell responses (10, 11). Because V $\beta$ 8.3 constitutes 30–50% of the WT D<sup>b</sup>NP<sub>366</sub><sup>+</sup>CD8<sup>+</sup> response and other subdominant V $\beta$ s (6) are also prominent, clonal diversity of D<sup>b</sup>NPM6A<sup>+</sup>CD8<sup>+</sup> T cells with  $\approx$ 2 prominent TCRV $\beta$ s and an average of  $\approx$ 3 clonotypes per V $\beta$  is lower than the WT D<sup>b</sup>NP<sub>366</sub><sup>+</sup>CD8<sup>+</sup> response. Analysis of the average number of clonotypes per mouse showed that, taking into account all V $\beta$ s analyzed, D<sup>b</sup>NPM6A elicited an average of 9.0 (1<sup>o</sup>) and 5.0 (2<sup>o</sup>) clonotypes per mouse. Relatively few clonotypes were found repeatedly in >1 mouse. Thus, this response (unlike D<sup>b</sup>NP<sub>366</sub>) is not public in character. Interestingly, although TCR diversity per 1 individual mouse was  $\approx$ 2 lower for





within the population) is a little more limited in range than that found for the WT D<sup>b</sup>NP<sub>366</sub> response, although the differential is at most 2-fold and the results conform with the earlier conclusion that flat and featureless pMHC–TCR interfaces (p6A is less prominent than p6M) tend to recruit a restricted TCR repertoire (10), although excessively “bulged” epitopes can be also associated with very limited TCR usage (17, 18). This further indicates that the public versus private character of a selected TCR repertoire is irrelevant when it comes to response magnitude and immunodominance hierarchies but is important for TCR diversity within the population. There must presumably be a sufficient number of “naïve” TCRs to select into a response, but whether these are more individualized or shared between mice may be of no consequence. Thus, although the topology of the pMHC determines the diversity of the TCR repertoire, the public or private character of that repertoire is determined by the availability of naïve clonotypes in all of the animals.

Analysis of virus growth characteristics indicated that the NPM6A substitution did not diminish viral fitness, and thus the amount of NP produced during the infectious process. The influenza virus NP is a key and abundant structural protein (7). Given that D<sup>b</sup>NPM6A generates a response that is of equivalent magnitude to that long recognized for D<sup>b</sup>NP<sub>366</sub> it seems reasonable to argue that the M6A change had no effect on the antigen-processing pathway. Thus, we are left with the conclusion that, if the protein/peptide load is equivalent for 2 different pMHC epitopes, then the relationship will be maintained for the response magnitude and the resultant, numerical immunodominance hierarchy. The diminished TCR/pMHC avidity profile associated with the NPM6A change indicates either that TCR avidity has no effect on response magnitude or, alternatively, that both interactions exceed a necessary threshold. The higher (with respect to D<sup>b</sup>NP<sub>366</sub>) TCR/pMHC avidity associated with recognition of D<sup>b</sup>PA<sub>224</sub> is also correlated with a different “functional avidity” profile (more TNF- $\alpha$  and IL-2 expression). Although TCR avidities varied for D<sup>b</sup>NP<sub>366</sub> and D<sup>b</sup>NPM6A, the strength of the TCR/pMHC interaction was evidently sufficient in both cases to promote an effective cytokine and CTL response. The relative efficacies might have changed if, for example, the mutant had shown the “higher” TCR/pMHC avidity profile associated with D<sup>b</sup>PA<sub>224</sub>.

The present results also emphasize that useful insights into the nature of relative epitope quality, or “fitness”, are unlikely to emerge from experiments that use only TCR-transgenic mice. Under those conditions there is, of course, no alternative TCR repertoire available for selection. We might also ask, though, whether a new TCR repertoire to a spontaneously arising mutant virus can emerge effectively during the course of infection with a persistent virus like HIV (13). If a preexisting, primed TCR memory pool to common mutations generated by the virus was to be elicited by preemptive vaccination, would this act to promote the control of persistent viruses or work against the emergence of tumor variants?

In conclusion, these experiments with the NPM6A mutation highlight that achieving an effective antigen dose is likely to be important for CD8<sup>+</sup> T cell vaccination and/or immunotherapy protocols. What is more intriguing, though, is that the results presented here suggest that immunization with at least some of these mutants will establish effective CD8<sup>+</sup> T cell memory. It may thus be possible to prime against the emergence of commonly selected mutants and thus diminish the possibility of virus (or tumor) escape.

## Methods

**Mice and Viral Infection.** C57BL/6J mice at 6 weeks of age were lightly anesthetized by methoxyfluorane inhalation and infected i.n. with 10<sup>4</sup> plaque-forming units (pfu) of the HKx31 (H3N2; HK) influenza A viruses in 30  $\mu$ L of PBS. Memory mice were primed i.p. with 1.5  $\times$  10<sup>7</sup> pfu of the serologically distinct

PR8 (H1N1) influenza A viruses that share the internal NP and PA proteins of HK. Mice were challenged i.n. with the HK viruses 6 weeks later to generate a secondary response.

**Generation and Titration of Recombinant Viruses.** Recombinant viruses were produced by an 8-plasmid reverse genetics system. A single amino acid mutation (M6A) was introduced into the NP<sub>366</sub> peptide, ASNENMETM, by PCR (primers available on request). PCR products were digested with BsmB1 and ligated into pHW2000 vector. The recombinant HK and PR8 viruses (HK-NPM6A and PR-NPM6A) were rescued after transfection of 8 plasmids encoding influenza segments into 293T and MDCK cells. Viruses were grown in embryonated eggs, and viral titers were determined by plaque assay.

**Tissue Sampling and Cell Preparation.** Spleen and BAL were recovered from mice at acute phases of 1<sup>o</sup> (day 10) and 2<sup>o</sup> (day 8) infections. Spleens were enriched for CD8<sup>+</sup> T cells by using anti-mouse IgG and IgM antibodies (Jackson ImmunoResearch Laboratories). Lungs were sampled on days 3, 6, 7, 8, or 9 after infection and homogenized, and the virus-containing supernatant was harvested. Infectious virus in lung supernatants was determined by plaque assay on MDCK cells.

**Tetramer Staining and Tetramer Dissociation Analysis.** CD8<sup>+</sup> T cells were stained with the D<sup>b</sup>NP<sub>366</sub>, D<sup>b</sup>PA<sub>224</sub>, or D<sup>b</sup>-NPM6A tetramers conjugated to Streptavidin-phycoerythrin (PE) (Molecular Probes) for 60 min at room temperature. Cells were washed twice in FACS buffer (10% BSA/0.02% NaAz in PBS), stained with a FITC-conjugated mAb to CD8 $\alpha$  (BD Biosciences Pharmingen) for 30 min on ice, washed, and analyzed by flow cytometry. As a measure of TCR avidity, splenic T cells were used in tetramer dissociation assay (4). After staining with tetramer, T cells were washed and incubated with anti-H2D<sup>b</sup> antibody at 5  $\mu$ g/mL to prevent tetramer rebinding. Cells were always stained and washed in the presence of NaAz to prevent tetramer internalization. Loss of tetramer<sup>+</sup>CD8<sup>+</sup> T cells at particular time points was calculated compared with tetramer staining at  $t = 0$  min.

**ICS.** Cells were stimulated with NP<sub>366</sub>, PA<sub>224</sub>, PB1<sub>703</sub>, or an array of NP-mutant peptides for 5 h in 200  $\mu$ L of cRPMI medium containing 1  $\mu$ g/ml Golgi-Plug (BD Biosciences Pharmingen), washed in FACS buffer, and stained with a PerCP-Cy5.5 conjugated mAb to CD8. Cells were fixed, permeabilized with a BD Cytotfix/Cytoperm kit, stained with mAbs to IFN- $\gamma$  (FITC), TNF- $\alpha$  [allophycocyanin (APC)], and IL-2 (PE) (BD Biosciences Pharmingen) for 30 min, washed, and analyzed by flow cytometry.

**Ex Vivo <sup>51</sup>Cr Release Assay.** Effector T cells were prepared from spleens of mice infected with HK or HK-NPM6A viruses. Cells were diluted across a range of effector-to-target ratios. EL-4 targets were pulsed with 1  $\mu$ M of NP<sub>366</sub> or NPM6A peptides and 750  $\mu$ Ci <sup>51</sup>Cr (Amersham Biosciences) for 1 h at 37  $^{\circ}$ C, washed, and adjusted to 10<sup>5</sup> cells per ml. One-hundred microliters was dispensed into plates, and effectors and targets were incubated for 4 h at 37  $^{\circ}$ C. Supernatants were transferred to Lumina plates (Packard Instruments). Gamma irradiation was measured, and percentage of specific lysis was calculated.

**Hybridoma LacZ Assay.** LacZ-inducible T cell hybridomas specific for NP<sub>366</sub> peptide (9) were dispensed into 96-well plates together with 5  $\times$  10<sup>5</sup> naïve splenocytes. Cells were cultured in the presence of NP<sub>366</sub> or NP mutant peptides at 10<sup>-6</sup> M for 18 h at 37  $^{\circ}$ C. Cells were washed, fixed, and incubated with X-Gal for 16 h at 37  $^{\circ}$ C. LacZ<sup>+</sup> hybridomas were counted.

**TCR Repertoire Analysis.** CD8<sup>+</sup> T cell populations were stained with the D<sup>b</sup>NPM6A tetramer, then anti-CD8-FITC. Cells were isolated by using a MoFlo sorter (Cytomation). D<sup>b</sup>NPM6A<sup>+</sup>CD8<sup>+</sup> T cells were sorted, and RNA was prepared by using TRIzol (Invitrogen). cDNA was reverse-transcribed with an Omniscript RT kit (Qiagen). PCR products were cloned into pCR2.1-TOPO (Invitrogen). Colonies containing inserts were sequenced on an Applied Biosystems Prism 3700 sequence analyzer.

**Protein Expression, Purification, Crystallization, and Structure Determination.** H2-D<sup>b</sup> and  $\beta$ 2-microglobulin molecules were expressed in *Escherichia coli* as inclusion bodies, refolded with the NP-M6A peptide, and purified (19, 20). D<sup>b</sup>NPM6A complex crystals were obtained at 7.4 mg/mL by the hanging-drop vapor diffusion technique at 20  $^{\circ}$ C. Crystals were grown with a reservoir containing 0.1 M potassium thiocyanate, 30% PEG 2000 (wt/vol). The crystals belong to space group P1, and the unit cell dimensions were consistent with 2 molecules per asymmetric units (Table S3). The crystals were flash-frozen to

a temperature of 100 K before data collection in-house on a R-Axis IV++ detector. Data were processed and scaled with the XDS (21). The crystal structure was solved using the molecular replacement method. The search probe used to solve the structure was the structure of mouse MHC class I H2-D<sup>b</sup> minus the peptide (Protein Data Bank ID code 1YN7) (10). The progress of refinement was monitored by the  $R_{\text{free}}$  value with neither a sigma nor a low-resolution cutoff being applied to the data. The refinement protocol used includes several cycles of refinement with REFMAC followed by manual model rebuilding with the O program (22). Refinement statistics are in Table S3.

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