A virus-specific CD8$^+$ T cell immunodominance hierarchy determined by antigen dose and precursor frequencies

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Immunodominance hierarchies are a substantial, but poorly understood, characteristic of CD8$^+$ T cell-mediated immunity. Factors influencing the differential responses to the influenza A virus nucleoprotein ($NP_{366–374}$) and acid polymerase ($PA_{224–233}$) peptides presented by $H2D^b$ have been analyzed by disabling (N5→Q substitution) these peptides in their native configuration, then expressing them in the viral neuraminidase protein. This strategy of shifting epitopes within the same viral context resulted in an apparent equalization of D^bNP_{366} [epitope consisting of viral nucleoprotein (NP) amino acid residues 366–374 complexed with the $H2D^b$ MHC class I glycoprotein] and D^bPA_{224} ($H2D^b+PA_{224–233}$) epitope abundance after direct infection *in vitro* and induced reproducible changes in the magnitude of the D^bNP_{366}- and D^bPA_{224}-specific T cell subsets generated after infection of mice. Comparison of D^bNP_{366}- and D^bPA_{224}-specific CD8$^+$ T cell responses induced from the native configuration and from the viral neuraminidase stalk demonstrated that the size of both primary and secondary responses is influenced by relative epitope levels and that, at least after secondary challenge, the magnitude of responses is also determined by CD8$^+$ T cell precursor frequency. Thus, this immunodominance hierarchy is a direct function of antigen dose and T cell numbers.

influenza A virus

Effector CD8$^+$ T cells specific for epitopes comprised of viral peptides bound to MHC class I glycoproteins (pMHC1) are required for efficient, acute control of the infectious process (1–3). Characteristically, virus-specific CD8$^+$ T cells recognize relatively few of the many possible pMHC1 combinations (4), whereas individual populations targeted to one or another pMHC1 complex vary in magnitude such that reproducible immunodominance hierarchies can be identified after primary or secondary challenge (4). Although the phenomena of epitope selectivity and differential prominence have been known for years, the underlying mechanisms are still unclear. Among the likely influences are relative protein abundance, differential antigen processing, efficiency of peptide binding to MHC1, variations in CD8$^+$ T cell precursor frequencies, and "immunodomination," whereby prominent CD8$^+$ T cell specificities suppress "minor" responses (4–16). How these various factors balance out to determine immunodominance hierarchies in a normal immune response is far from clear.

An intriguing immunodominance hierarchy is found for the CD8$^+$ T cell responses to the nucleoprotein NP_{366} and acid polymerase PA_{224} peptides presented by $H2D^b$ in C57BL/6J mice (B6 mice) infected with influenza A virus. Although the primary CD8$^+$ T cell responses to D^bNP_{366} [epitope consisting of viral nucleoprotein (NP) amino acid residues 366–374 complexed with the $H2D^b$ MHC class I glycoprotein] and D^bPA_{224} ($H2D^b+PA_{224–233}$) are of equivalent size, the D^bNP_{366}-specific set achieves a much greater magnitude after secondary challenge, constituting ≈80–90% of all influenza-specific CD8$^+$ T cells (5, 17–19). This divergence in the extent of clonal expansion for D^bPA_{224} and D^bNP_{366} after secondary virus challenge could reflect differences in the associated antigen-presenting cell (APC) profiles. Although D^bNP_{366} is readily detected on both dendritic cells (DCs) and non-DCs recovered directly from influenza virus-infected B6 mice, efficient D^bPA_{224} expression is apparently restricted to the DC population (10). The equivalent, primary D^bNP_{366}- and D^bPA_{224}-specific responses might thus be a consequence of the naïve T cell requirement for DC stimulation, whereas D^bNP_{366}-specific T cell dominance in the secondary response reflects the increased range of D^bNP_{366} presentation and the less stringent requirement for DC stimulation. However, although primary responses have long been thought to be induced solely by DCs, the "professional" APCs, recent experiments suggest that this may also be true after secondary challenge (20). Furthermore, there is evidence to suggest that even within the DC population the D^bNP_{366} epitope is presented at higher levels than D^bPA_{224} (9).

The present analysis investigates the contributions of T cell numbers and epitope presentation levels in the influenza virus-specific cellular immune response. By expressing the NP_{366} and PA_{224} peptides within the influenza virus such that differences in D^bNP_{366} and D^bPA_{224} epitope presentation are eliminated, we have demonstrated clear roles for antigen load and precursor T cell numbers in determining the D^bNP_{366} and D^bPA_{224} CD8$^+$ T cell immunodominance hierarchies.

Results

Factors Determining the Effective Antigen Dose. Intranasal (i.n.) challenge with influenza A viruses leads to an infectious process that is effectively limited *in vivo* to the superficial epithelial layer of the mouse respiratory tract because of a restricted distribution of a host enzyme required to cleave the viral hemagglutinin (H) molecule (21, 22). Effective influenza A virus antigen load *in vivo* is therefore a direct consequence of replicative infection in the lung after i.n. exposure or of nonreplicative infection in the various APC populations subsequent to i.n. or i.p. challenge. Measurement of lung virus titers at 24 h after i.n. infection of naïve B6 mice with 200 plaque-forming units (pfu) of the wild-type (wt) and the engineered NA^{NP} or NA^{PA} viruses indicated that introduction of the NP_{366} or PA_{224} peptide into the viral neuraminidase (NA) did not substan-

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Abbreviations: APC, antigen-presenting cell; B6, C57BL/6J mice; DC, dendritic cell; BmDC, bone marrow DC; N or NA, viral neuraminidase; NP, viral nucleoprotein; i.n., intranasal(ly); PA, viral acid polymerase; PE, phycoerythrin; pfu, plaque-forming unit; PR8, A/PR/8/34 H1N1 influenza virus; pMHC1, peptides bound to MHC class I glycoprotein; HKx31 virus, A/HKx31 H3N2 influenza virus; TCR, T cell receptor; μMT mice, Ig$^{-/-}$ mice; wt, wild type.

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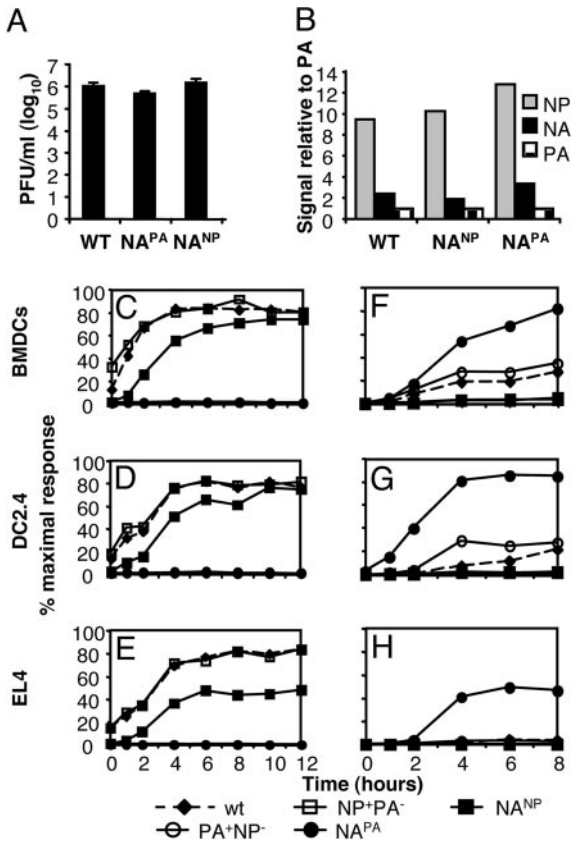

Fig. 1. Insertion of the NP_{366} or PA_{224} peptides into the viral NA has no effect on virus replication in the lung but alters D^bNP_{366} and D^bPA_{224} presentation levels on APCs. Four or five B6 mice were infected i.n. with 200 pfu of PR8 wt, NA^{NP}, or NA^{PA} viruses, and lungs were sampled for virus titration 24 h later (*A*). EL4 cells were infected with PR8 wt, NA^{NP}, or NA^{PA} viruses for 3 h, and RNA was extracted 5 h later. After reverse transcription, cDNA was amplified by real-time PCR by using SYBR chemistry and NP-, PA-, and NA-specific oligonucleotides. Data are shown as amount of signal relative to PA (*B*). BmDCs, DC2.4 cells, and EL4 cells were infected *in vitro* with the designated PR8 recombinant or wt viruses for 1 h at 37°C. After infection, APCs were added to short-term D^bNP_{366}- or D^bPA_{224}-specific CTL lines in the presence of brefeldin A at the times shown (*C–H*). The T cells were harvested 4 h later and analyzed for expression of CD8α and IFN-γ. Results are plotted as the percentage of the maximal response determined by stimulating CTL lines with EL4 cells at saturating peptide doses. Shown are the D^bNP_{366}-specific (*C–E*) and D^bPA_{224}-specific (*F–H*) CTL responses to epitopes presented by BmDCs (*C* and *F*), DC2.4 cells (*D* and *G*), and EL4 cells (*E* and *H*) after infection with the indicated viruses.

tially modify the capacity of these viruses to replicate in the respiratory tract (Fig. 1*A*).

By using quantitative real-time PCR, the relative levels of NP, viral acid polymerase (PA), and NA mRNA were determined after *in vitro* infection of EL4 cells with the A/PR/8/34 H1N1 influenza virus (PR8) wt, NA^{NP}, and NA^{PA} influenza A viruses. This analysis served to ensure that insertion of novel peptide sequences into the NA did not disrupt transcription and provided insight into the likely abundance of NP_{366} and PA_{224} from the native protein versus the engineered NA stalk, which is likely to be a key factor determining peptide availability to the MHC class I glycoproteins. Similar ratios were observed for the NP, NA, and PA transcripts in all three infections (Fig. 1*B*), indicating that neither the introduction of additional peptide sequence into NA nor N5Q substitution in the native NP and PA (NP^- and PA^-) perturbs normal transcription profiles. Given that the NA mRNA levels were 2- to 4-fold higher than those for PA but substantially (3- to 5-fold) lower than those for NP, it is likely that the insertion of NP_{366} or PA_{224} into NA leads to lower and higher abundance, respectively, of these peptides relative to that from the wt viruses. Also, the NP_{366} and the PA_{224} peptides are presumably produced at similar levels after infection with the NA^{NP} or NA^{PA} viruses, reflecting the amount of NA made.

After infection with wt influenza A viruses, the D^bNP_{366} epitope can be detected on a spectrum of cell types, whereas efficient D^bPA_{224} presentation is restricted to DCs (9, 10). Furthermore, D^bNP_{366} appears before D^bPA_{224} on the DC plasma membrane, measured kinetically by using brefeldin A to block export of newly synthesized pMHC1 complexes from the endoplasmic reticulum (7). This assay was used to determine whether the profiles of epitope expression were modified for the NA^{NP} or NA^{PA} viruses (Fig. 1 *C–H*). As expected, whereas the presence of the NP_{366} peptide in the native context (NP^+PA^-) led to equivalent levels of D^bNP_{366} presentation on all three APC populations (Fig. 1 *C–E*), viruses expressing the "native" PA_{224} peptide (wt, PA^+NP^-) induced detectable D^bPA_{224} on the DC cells [bone marrow DC (BmDC) and DC2.4] but not on the non-DC EL4 targets (Fig. 1, compare *H* with *F* and *G*). Thus, relocating PA_{224} to the NA stalk (NA^{PA}) led to measurable D^bPA_{224} presentation on the nondendritic EL4 cells (Fig. 1*H*). Furthermore, both the rate and overall level of D^bPA_{224} expression on all three APC populations were significantly enhanced when they were infected with the NA^{PA} rather than the PA^+NP^- or wt viruses (Fig. 1 *F–H*).

Conversely, the amounts of D^bNP_{366} were uniformly decreased across all APC populations when the peptide was expressed in the NA rather than in the native position (Fig. 1 *C–E*). Although the presentation of D^bNP_{366} by the NP^+PA^-- and NA^{NP}-infected DCs appeared equivalent at 12 h, this was most likely because of saturation of T cell activation and would therefore not necessarily be a reflection of equivalent presentation (Fig. 1 *C* and *D*). In summary, situating the NP_{366} and PA_{224} peptides in the same protein context clearly led to an equilibration in the rate, overall level, and range of D^bNP_{366} and D^bPA_{224} expression for DCs and non-DC APCs. Does this in turn modify the immunodominance hierarchy in virus-infected mice?

Quantitation of $CD8^+$ T Cell Responses After Primary Infection. Naïve B6 mice were infected i.p. with 1.5×10^7 pfu of the NA^{NP}, NP^+PA^-, NA^{PA}, and PA^+NP^- viruses. The $CD8^+$ T cell responses to D^bNP_{366} and D^bPA_{224} were then measured by tetramer staining of splenocytes at the peak of the response on day 10 after infection (Fig. 2*A*). The numbers of D^bNP_{366}-specific $CD8^+$ T cells were greatly decreased ($P < 0.00001$) after infection with the NA^{NP} virus compared with the NP^+PA^- virus (Fig. 2*A*). Conversely, the size of the D^bPA_{224}-specific $CD8^+$ T cell population induced by the NA^{PA} virus was substantially increased ($P < 0.001$) relative to the results for the PA^+NP^- challenge (Fig. 2*A*). Both sets of findings correlated with the profiles of protein expression and antigen presentation inferred from the RT-PCR (Fig. 1*B*) and IFN-γ stimulation experiments (Fig. 1 *C–H*).

Comparison of mice infected with either the NP^+PA^- or PA^+NP^- viruses indicated that the D^bNP_{366}-specific response was, on average, two times greater than that induced by the D^bPA_{224} epitope (first and third sets of data in Fig. 2*A*). However, infection with the NA viruses reversed this relationship to give a D^bPA_{224}-specific response that was five times larger (second and fourth sets of data in Fig. 2*A*). Thus, when these peptides are identically situated within the NA protein and consequently presented at equivalent levels (Fig. 1), the primary D^bPA_{224}-specific $CD8^+$ T cell response seems to have a significant advantage ($P > 0.0001$) over that directed to D^bNP_{366}.

The diminished size of the acute D^bNP_{366}-specific response (Fig. 2*A*) induced by the NA^{NP} virus was maintained into memory (Fig. 2*B*), being significantly smaller than the $CD8^+D^bNP_{366}{}^+$ and $CD8^+D^bPA_{224}{}^+$ T cell counts at day 55 after priming with the other three viruses (Fig. 2*B*). However, the smaller differences found early (Fig. 2*A*) after priming among the NP^+PA^-, PA^+NP^-, and

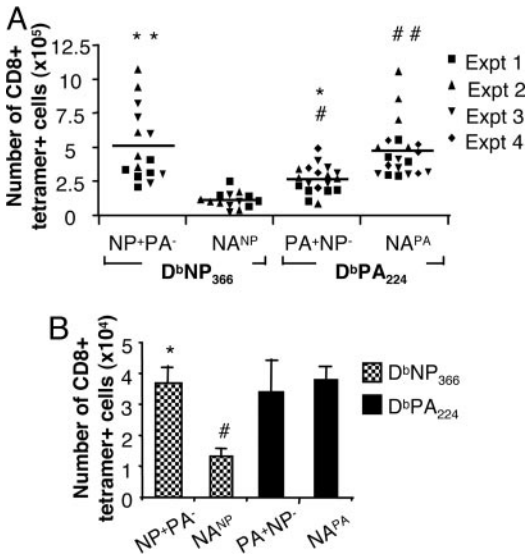

Fig. 2. The magnitude of acute and memory D^bNP_{366}- and D^bPA_{224}-specific responses is altered after NA^{NP} and NA^{PA} viral challenge. B6 mice were infected i.p. with NP^+PA^-, NA^{NP}, PA^+NP^-, or NA^{PA} influenza A viruses, and splenocytes were sampled 10 days (*A*) or 55 days (*B*) later. Cells were stained with the D^bNP_{366}-PE or D^bPA_{224}-PE tetramers followed by anti-CD8α-FITC. *, $P < 0.0005$; **, $P < 0.00001$ comparing NP^+PA^- with NA^{NP} or PA^+NP^- with NA^{PA}. #, $P < 0.001$; ##, $P < 0.00001$ comparing NP^+PA^- with PA^+NP^- or NA^{NP} with NA^{PA} (Student's *t* test). The results in *A* are shown for individual mice from four separate experiments. Data in *B* are mean ± SD for groups of five mice.

NA^{PA} viruses were not sustained in the longer term (Fig. 2*B*). The numbers of virus-specific $CD8^+$ T cells in the spleen decreased overall by a factor of ≈10 times during this interval (Fig. 2, compare *A* with *B*), which presumably led to some equilibration in population size.

T Cell Repertoire Analysis After Primary Infection. Given that altering the protein context of the NP_{366} and PA_{224} peptides significantly modifies both the antigen presentation characteristics for the D^bNP_{366} and D^bPA_{224} epitopes (Fig. 1) and the magnitude of acute virus-specific $CD8^+$ T cell responses (Fig. 2), it seemed reasonable to ask whether there was any consequent change in the D^bNP_{366}- and D^bPA_{224}-specific T cell receptor (TCR) repertoires, which have been extensively characterized after wt virus infection (23, 24).

Single-cell RT-PCR analysis was performed on $CD8^+$ T cells from B6 mice primed i.p. 10 days previously with the NP^+PA^-, NA^{NP}, PA^+NP^-, and NA^{PA} viruses (Table 1). Initial analysis of Vβ chain usage in D^bNP_{366}- and D^bPA_{224}-specific populations showed characteristic Vβ8.3 and Vβ7 biases, respectively, regardless of whether the epitope-specific populations were induced by the native or NA viruses (Table 1). Determining the CDR3β sequences of >200 individual $CD8^+$tetramer$^+$ T cells from each group indicated that modifying the character of antigen presentation, and the consequent magnitude of the response to D^bNP_{366} and D^bPA_{224}, did not alter the character of TCR repertoire selection (Table 1). Providing more (D^bPA_{224}) or less (D^bNP_{366}) antigen by priming with the NA^{PA} and NA^{NP} viruses had no effect on either CDR3β phenotype or diversity as measured by clonotype number (Table 1). The composition of the TCR repertoire thus seems to be independent of the size of the response after primary challenge.

Effect on the Recall Response. Primary infection of naïve mice with the NA viruses, relative to the native viruses, induced significantly lower D^bNP_{366}-specific and equivalent D^bPA_{224}-specific memory numbers (Fig. 2*B*). To what extent is this reflected in the magnitude of the recall response after secondary challenge? To analyze the secondary influenza A virus-specific $CD8^+$ T cell response we typically prime i.p with the PR8 (H1N1) influenza A viruses, rest the mice for at least a month, then infect i.n. with the relatively avirulent A/HKx31 H3N2 influenza virus (HKx31 virus) that shares the PR8 internal components (including NP and PA) but carries different (H3N2) surface glycoproteins. The H3N2→H1N1 cross-challenge ensures that there is no diminution of the input dose as a consequence of antibody-mediated neutralization (25). Attempts to express the NP_{336} and PA_{224} peptides in the NA of the H3N2 virus have been unsuccessful, so it has not been possible to follow this protocol. Consequently, neutralization of the challenge virus was avoided by using $Ig^{-/-}$ μMT mice for homologous i.p. prime/boost studies with the PR8 viruses.

Looking first at the D^bNP_{366}-specific response in μMT mice primed with the NP^+PA^- virus, the lower antigenicity of the NA^{NP} virus was clearly associated with a smaller recall response (compare first two bars in Fig. 3*A*). Similarly, those primed with the NA^{NP} virus, which induces a lower level of $CD8^+$ T cell memory in $Ig^{+/+}$ B6 mice (Fig. 2*B*), showed a greatly diminished secondary response when challenged with the "high-dose" NP^+PA^- virus (first and third bars in Fig. 3*A*), indicating that this defect in precursor numbers was not overcome by increasing the subsequent antigen load. The results for the NA^{NP}→NA^{NP} challenge did not give the predicted result, but only two mice survived in this group (fourth bar in Fig. 3*A*). The direct correlation between epitope dose and the extent of subsequent clonal expansion was, however, very clear for μMT mice primed with either the PA^+NP^- virus or the NA^{PA} virus, because challenge with the NA^{PA} virus induced a larger D^bPA_{224}-specific response, irrespective of the priming virus (Fig. 3*B*). Unlike the situation for D^bNP_{366}-specific T cells, the priming regime had no obvious effect on the D^bPA_{224}-specific response (Fig. 3*B*), but the numbers of memory T cells found in B6 mice at day 55 after the initial exposure to the PA^+NP^- or NA^{PA} virus were not significantly different (Fig. 2*B*). Together (Fig. 3), these results indicate that the magnitude of a secondary response is determined by both the relative availability of memory T cells before challenge and the effective antigen dose during challenge. Whereas the anomalous result seen for the groups primed with NA^{NP} is most likely due to the limited number of mice in one group, it is possible that a high-dose antigen persistence in μMT mice may be inducing

Table 1. Relative diversity of D^bNP_{366}- and D^bPA_{224}-specific TCRβ repertoires

Mouse	No. of TCRs sequenced	Modal CDR3β length, aa	Vβ preference* (% of $CD8^+$ tetramer$^+$ cells)	Jβ preference	Total no. of clonotypes		No. of clonotypes per mouse	
					aa	Nucleotide	aa	Nucleotide
NP^+PA^-	299	9	8.3 (51.1 ± 20.1)	2.2	18	24	6 ± 1.6	7.2 ± 2.2
NA^{NP}	207	9	8.3 (50.2 ± 23.6)	2.2	20	24	6 ± 4.1	7.4 ± 4.0
PA^+NP^-	283	6	7 (53.4 ± 6.4)	2.6, 1.1	109	—	24.8 ± 3.5	—
NA^{PA}	299	6	7 (57.9 ± 9.9)	2.6, 1.1	98	—	23.8 ± 7.3	—

Data are from five mice 10 days after primary i.p. immunization with 1.5×10^7 pfu of designated PR8 viruses.
*Preference for Vβ8.3 (D^bNP_{366}) or Vβ7 (D^bPA_{224}).

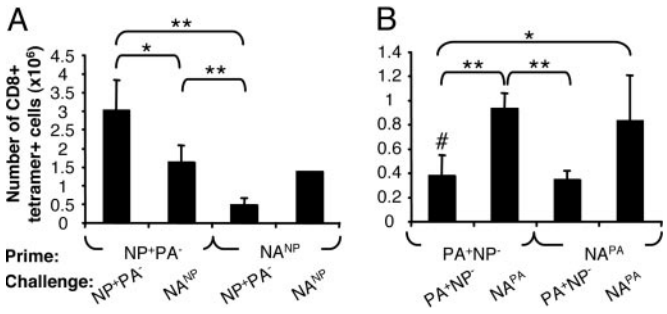

Fig. 3. Consequences of priming and boosting with recombinant viruses in B6 μMT mice. Mice were primed i.p. with 1.5×10^7 pfu of the NP^+PA^-, NA^{NP}, PA^+NP^-, or NA^{PA} influenza A viruses and then challenged i.p. 5 weeks later with the indicated PR8 viruses (1.5×10^7 pfu). Splenocytes were sampled 8 days after secondary challenge, and T cells were stained with the D^bNP_{366}-PE (*A*) or D^bPA_{224}-PE (*B*) tetramers followed by anti-CD8α-FITC. Shown are the mean numbers of $CD8^+$tetramer$^+$ cells for, generally, groups of three to five mice. *, $P < 0.05$; **, $P < 0.01$ (Student's *t* test).

apoptosis in the NP^+PA^- group, although this was not observed in mice receiving the PA_{224}-expressing viruses.

The μMT mice (Fig. 3) have small spleens and are not particularly robust, so the comparison was continued (Fig. 4) for conventional B6 mice primed with either the engineered NP^+PA^- and PA^+NP^- (Fig. 4*A*), or the wt (Fig. 4*B*) HKx31 (H3N2) viruses, then challenged i.n. with the PR8 viruses expressing NP_{366} or PA_{224} in the native protein (NP^+PA^-, PA^+NP^-) or in the NA configuration (NA^{NP}, NA^{PA}). The numbers of D^bNP_{366}- and D^bPA_{224}-specific memory T cells induced by these different immunization strategies are similar (Fig. 2*B*), but, after challenge with the native context viruses, the recall response to D^bNP_{366} is generally at least five times higher than the D^bPA_{224}-specific response (first and third bars in Fig. 4) (17–19, 26). Again, the magnitude of the secondary response in the spleen on day 8 was directly related to the antigen load inferred from the *in vitro* studies (Fig. 1). The numbers of D^bNP_{366}- and D^bPA_{224}-specific T cells induced by the NA^{NP} and NA^{PA} viruses were essentially equivalent (second and fourth bars in Fig. 4), whereas the counts for mice challenged with the NP^+PA^- virus were higher (first and second bars in Fig. 4) and those challenged with the PA^+NP^- virus were lower (third and fourth bars in Fig. 4) than those found after challenge with the NA^{NP} and NA^{PA} viruses, respectively. The relevant data set for the μMT mice (Fig. 3) is repeated for comparison (open bars in Fig. 4*A*). Together (Figs. 3 and 4), these results demonstrate that, unlike the situation found in the primary response, controlling the protein context in a way that equalizes the abundance and processing efficiency of the D^bNP_{366} and D^bPA_{224} peptides results in a marked equalizing of the recall responses. The immunodominance hierarchy (17–19, 26) found for the D^bNP_{366}- and D^bPA_{224}-specific responses after secondary challenge is thus very much a function of antigen load.

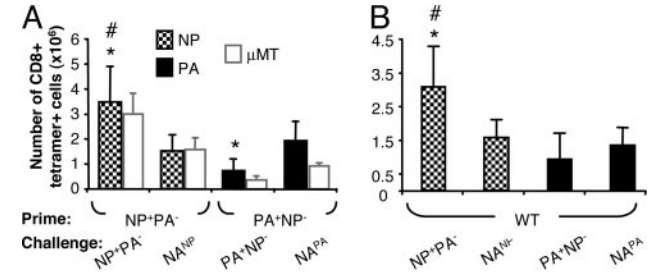

Fig. 4. The magnitude of secondary recall is influenced by the level of epitope presentation. B6 mice were primed i.p. with 1.5×10^7 pfu of the HKx31 NP^+PA^-, PA^+NP^- variants (*A*) or with wt HKx31 virus (*B*), then challenged i.n. 6–8 weeks later with 200 pfu of the PR8 NP^+PA^-, NA^{NP}, PA^+NP^-, or NA^{PA} viruses. Splenocytes were harvested 8 days later and stained with D^bNP_{366}-PE or D^bPA_{224}-PE tetramers followed by anti-CD8α-FITC. Shown are the mean splenic T cell numbers for groups of four to five mice. For comparison, data from the corresponding groups of B6 μMT mice (see Fig. 3) are shown in *A* as open bars. *, $P < 0.05$ comparing NP^+PA^- with NA^{NP} challenge or PA^+NP^- with NA^{PA} challenge. #, $P < 0.01$ comparing NP^+PA^- with PA^+NP^- challenge (Student's *t* test).

Discussion

The present NA substitution strategy modifies both the antigen dose and (for D^bPA_{224}) the spectrum of APCs that naïve and memory T cells specific for native viral epitopes encounter during the course of a viral pneumonia. To our knowledge, this represents the first study in which peptide protein context has been switched without altering the pathogenesis of an otherwise unmanipulated, virus-induced infectious process. The findings support a model wherein precursor frequency and epitope density are the prime determinants of the D^bNP_{366}/D^bPA_{224} immunodominance hierarchy after influenza virus infection.

Because this analysis of epitope presentation used direct infection, no useful conclusions can be reached concerning the effect of possible cross-presentation on epitope availability. The shifts in epitope presentation are, however, clearly related to changes in protein abundance, which might be expected to affect both cross-presentation and direct presentation. Furthermore, although the equivalent D^bNP_{366}-specific responses to NA^{NP}virus- and NP^+PA^- virus-infected cells at 12 h indicate that the level of T cell stimulation has been maximized, it is equally possible that this reflects the saturation of D^bNP_{366} presentation on the APC population. If this is the case, our results suggest that early (<12 h), high-level expression of NP is a particularly important determinant of response magnitude, either by means of direct presentation or by making larger amounts of protein available for cross-presentation.

Proposed determinants of immunodominance other than precursor frequency and antigen dose appear to be minimally relevant in determining the D^bNP_{366}/D^bPA_{224} immunodominance hierarchy. Although we cannot formally exclude the effects of differential peptide processing efficiency on establishing immunodominance hierarchies after wt infection, the D^bNP_{366} and D^bPA_{224} epitope presentation levels correlate well with differences in the amount of NA, NP, and PA protein produced, suggesting that, in this system, relative levels of direct epitope presentation are predominantly determined by the abundance of protein rather than peptide-processing efficiency. The NA^{NP} and NA^{PA} viruses express either NP_{366} or PA_{224} in NA while lacking both peptides (NP^-PA^-) in their native configurations. Previous experiments with single-knockout NP^+PA^- and PA^+NP^- viruses have demonstrated that eliminating the D^bNP_{366} epitope causes only a modest enhancement of the secondary D^bPA_{224}-specific response, whereas the removal of D^bPA_{224} has no discernable effect on $CD8^+$ D^bNP_{366}-specific T cell numbers (27, 28). Furthermore, the sizes of the independent D^bNP_{366} and D^bPA_{224} populations induced by the NP^+PA^- and PA^+NP^- viruses in this study correlate with the relative magnitudes of the D^bNP_{366} and D^bPA_{224} sets generated after wt virus infection (17–19, 26, 29). Thus, any competitive interaction, or immunodomination effect, has comparatively little consequence for these two responses. A recent study revealed a significantly higher binding avidity for D^b by the PA_{224} compared with the NP_{366} peptide (unpublished data), indicating that this also has little effect on immunodominance hierarchies in this system.

The acute responses, and the size of the resultant memory T cell pools, are essentially equivalent for D^bNP_{366} and D^bPA_{224} after the initial encounter with viruses expressing these two peptides in their native configurations. However, a clear $D^bPA_{224} \rightarrow D^bNP_{366}$ hierarchy is apparent after primary infection with the NA^{NP} and NA^{PA} viruses, whereas, in the secondary response, given comparable memory T cell numbers, the D^bNP_{366} and D^bPA_{224} sets achieve much the same magnitude after secondary NA^{NP} and NA^{PA}

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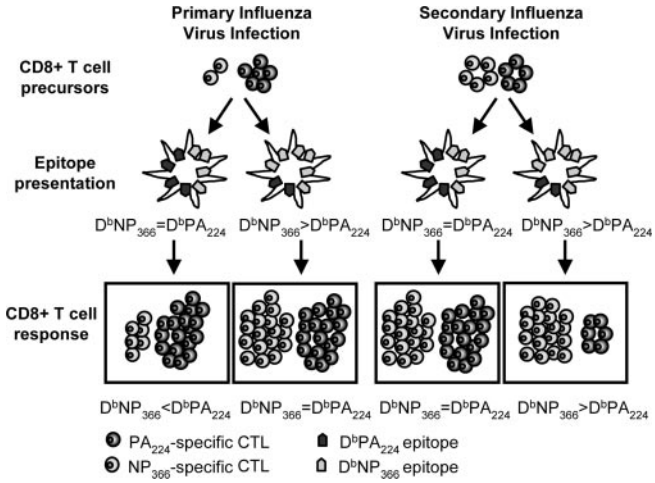

Fig. 5. A proposed model linking the contributions of precursor frequencies and epitope levels as determinants of CD8$^+$ T cell response magnitude, and thus immunodominance hierarchies, after influenza virus infection.

challenge. The difference in magnitude between the D^bNP_{366}- and D^bPA_{224}-specific populations in response to primary NA^{NP} and NA^{PA} immunization contradicts the model proposed by Crowe *et al.* (10) which suggests that equivalent primary responses to D^bNP_{366} and D^bPA_{224} after wt infection is due to equivalent presentation by DCs.

We propose a model in which the divergence between the consequences of primary and secondary infection is the result of a substantially larger naïve D^bPA_{224}-specific, compared with D^bNP_{366}-specific, precursor frequency. Combined with an overall higher density of D^bNP_{366} presentation on DCs after wt infection, this results in equivalent primary D^bNP_{366}- and D^bPA_{224}-specific responses (Fig. 5). Although we currently have no direct method for counting the naïve repertoire, this interpretation is supported by TCR sequence data (23, 24) showing greater TCR repertoire diversity for the D^bPA_{224}-specific population relative to the D^bNP_{366}-specific set. This idea that the naïve D^bPA_{224}-specific precursor frequency is higher is also supported by a separate mathematical analysis (S. Perlman, unpublished observations) and by the observation that the primary response to D^bPA_{224} peaks 1–2 days earlier than that to D^bNP_{366} (17–19, 26). Furthermore, our model suggests that, for the recall of memory, the presence of equivalent D^bNP_{366}- and D^bPA_{224}-specific memory CD8$^+$ T numbers combines with a relatively high level of D^bNP_{366} epitope presentation to generate the substantial D^bNP_{366}-specific T cell dominance observed after secondary challenge with wt virus (Fig. 5). A correlation between precursor frequency and response magnitude was also found for D^bNP_{366} after secondary challenge of the NA^{NP}-primed μMT mice, where the high-dose NP^+PA^- virus was unable to compensate for lower memory T cell numbers.

Because the mouse experiments used equivalent virus doses that caused viral protein synthesis without replicative infection (high titer i.p.) or induced comparable profiles of virus production in the respiratory tract (low titer i.n.), any alteration in the magnitude of the effective *in vivo* challenge may (at least for D^bNP_{366}) be a direct reflection of the change in epitope density on the surface of individual APCs rather than an increase or decrease in APC numbers. Even so, positioning the PA_{224} peptide in the NA stalk did modify the spectrum of cell types expressing the D^bPA_{224} epitope. However, *in vitro* infection with the NA^{PA} virus also caused a substantial increase in the level of epitope expression on DCs so, unlike the situation for D^bNP_{366}, where the spectrum of potential APC diversity remains constant, these two effects (epitope density vs. non-DC APCs) cannot be formally separated for the D^bPA_{224}-specific response. However, recent evidence suggests that both primary and secondary CD8$^+$ T cell responses are largely, if not totally, DC-dependent (20, 30). As such, non-DC presentation of D^bNP_{366} (or D^bPA_{224}) may have little influence on the immunodominance hierarchy.

Interestingly, the present results indicate that selection of particular TCRs does not depend on antigen dose, because there was no change in the spectrum of repertoires selected by epitopes derived from either the native context or the NA stalk, suggesting an inherent plasticity of epitope-specific repertoires. Recruitment of specific T cells into an epitope-specific response, and thus the composition of the epitope-specific TCR repertoire, is likely to reflect the character of the pMHC1 antigen. The "less featured" surface presented to the TCR by D^bNP_{366} is clearly "seen" by a smaller spectrum of clonotypes than the "more obtrusive" D^bPA_{224}, with its projecting arginine (31). In that sense, the pMHC1 epitope landscape that determines the diversity in selection of the TCR repertoire will influence the magnitude of the consequent immunodominance hierarchy after primary challenge by increasing the precursor frequency. However, the present analysis also suggests that any defect in the primary response associated with lower numbers of naïve precursors is readily overcome by increasing the effective antigen load. Although the composition of the responding T cell repertoires appears unchanged when induced by NA or wt context viruses, further studies would be required to determine whether the relative avidities of the epitope-specific populations are changed, because antigen dose has been shown by others to correlate inversely with T cell avidity (32, 33).

Materials and Methods

Mice and Tissue Sampling. Female B6 (H-2^b) mice were bred in the animal facility of the Department of Microbiology and Immunology at the University of Melbourne. Congenic μMT mice (34) were bred and housed at St. Jude Children's Research Hospital. Naïve mice (6–8 weeks old) were infected i.p. with 1.5×10^7 pfu of PR8 wt or recombinant influenza A virus, and those used to analyze the secondary response to recombinant virus infection were primed i.p. with 1.5×10^7 pfu of HKx31 or PR8 virus at least 6 weeks before i.p. (1.5×10^7 pfu) or i.n. (200 pfu) challenge with PR8.

Recombinant Viruses. Mutant, recombinant influenza A viruses were generated by using an eight-plasmid reverse genetics system described in refs. 35 and 36. Recombinant PR8 (H1N1) viruses expressing either the NP_{366} (ASNENMETM) or PA_{224} (SSLENFRAYV) peptide inserted into the NA stalk at amino acid position 42 (referred to as the NA^{NP} and NA^{PA} viruses, respectively) also contained single amino acid mutations (28) in both the native NP_{366} and PA_{224} epitopes (N5Q) to disrupt presentation (NP^-PA^-). Control viruses expressing either the NP_{366} or PA_{224} epitope in the native context (NP^+PA^- or PA^+NP^-, respectively) harbor the N5Q mutation in either the native PA_{224} or NP_{366} peptide (28) (see Table 2, which is published as supporting information on the PNAS web site).

Measuring Lung Viral Titers. Lungs were homogenized in 1 ml of RPMI medium 1640 containing 24 μg/ml gentamycin and 100 units/ml penicillin/streptomycin 24 h after i.n. infection with 200 pfu of wt, NA^{NP}, or NA^{PA} PR8 viruses, and titers (pfu/ml) were determined by plaque assay on Madin-Darby canine kidney cell monolayers (37).

Tetramer Staining. Virus-specific CD8$^+$ T cells were identified by using tetrameric complexes of $H2D^b$ and either the NP_{366} or PA_{224} peptide. Monomeric class I/peptide complexes were provided by the Tetramer Core Facility at St. Jude Children's Research Hospital and were tetramerized by using streptavidin-conjugated phycoerythrin (PE) or allophycocyanin (Molecular Probes). Cells were stained with PE- or allophycocyanin-conjugated D^bNP_{366} or

D^bPA_{224} tetramers (19, 26) for 1 h at room temperature, then with anti-mouse CD8α-FITC (53-6.7; Pharmingen) for 30 min on ice.

Measuring Viral mRNA Transcripts. Cultured EL4 cells were infected with the wt or recombinant NA^{NP} or NA^{PA} viruses for 3 h, washed, and incubated at 37°C for a further 5 h before total RNA extraction with TRIzol (Invitrogen). The mRNA transcripts were then reverse-transcribed by using oligo(dT), and the cDNA was treated with RNase before amplification by real-time PCR by using SYBR Green PCR master mix (Applied Biosystems) and NP-, PA-, or NA-specific oligonucleotides. Samples were run on an ABI 7700, and data were analyzed by using the ΔCT method to determine relative amounts of specific product.

Determining Epitope Expression Profiles by Intracellular Cytokine Staining. These experiments focused particularly on the APC characteristics of infected DCs. Primary DCs (BmDC) were grown from B6 femoral bone marrow by culture in granulocyte/macrophage colony-stimulating factor-enriched RPMI medium 1640 supplemented with 10% FCS for 7–8 days. The $CD8^+$ T cell lines used in this assay were generated by culturing splenocytes from primed B6 mice with peptide-pulsed splenocytes in the presence of IL-2 (10 units/ml). Viable T cells were harvested after one round of *in vitro* stimulation. Sensitivity of the D^bNP_{366}- and D^bPA_{224}-specific $CD8^+$ T cell lines was similar, as determined by peptide titration experiments. Aliquots of 10^5 BmDC, EL-4, or DC2.4 (DC-like cell line, H-2^b) cells were infected with the various PR8 viruses for 60 min at 37°C; then, at various time points thereafter, 10^5 peptide-specific $CD8^+$ T cells were added in conjunction with 10 mg/ml brefeldin A (Sigma-Aldrich) to "freeze" further antigen processing and presentation. The initial time point was designated $t = 0$ h. After a further 4 h, the T cells were harvested, then stored at 4°C until all samples were stained with a CyChrome anti-CD8a mAb at 4°C for 20 min, washed, fixed with 1% paraformaldehyde in PBS at room temperature for 20 min, and stained with anti-IFN-γ-FITC in 0.2% saponin (Sigma)/PBS. Viable $CD8^+$ T cells were analyzed for IFN-γ on a FACSCalibur (Becton Dickinson) by using FLOWJO software (Tree Star, Ashland, OR).

Isolation of Single $CD8^+$ T Cells, RT-PCR, and Sequencing. Lymphocytes were isolated with a MoFlo sorter (Cytomation, Fort Collins, CO) fitted with a Cyclone single-cell deposition unit. Single immune $CD8^+V\beta8.3^+D^bNP_{366}^+$ or $CD8^+V\beta7^+D^bPA_{224}^+$ T cells were sorted directly into a 96-well PCR plate (Eppendorf) containing 5 μl of cDNA reaction mix, and reverse transcription and PCR were performed as described in refs. 23 and 24. Negative controls were interspersed between the samples (1 in 10), and 50–80 cells were sorted per plate.

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