Supporting Information

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A

Sequence	Variant	Position 6 amino acid properties	H2-D^b Thermo-stability (°C)	Conserved^	Non conserved^	Viral escape variants Freq$^+$
ASNENMETM	wt	Hydrophobic	51.8 ± 0.7			
-----W---	M6W	Hydrophobic aromatic	58.8 ± 0.9		X	-
-----Y---	M6Y	Polar aromatic			X	-
-----I---	M6I	Hydrophobic aliphatic	58.6 ± 1.0	X		18/35
-----T---	M6T	Polar	56.2 ± 0.5		X	1/35

B

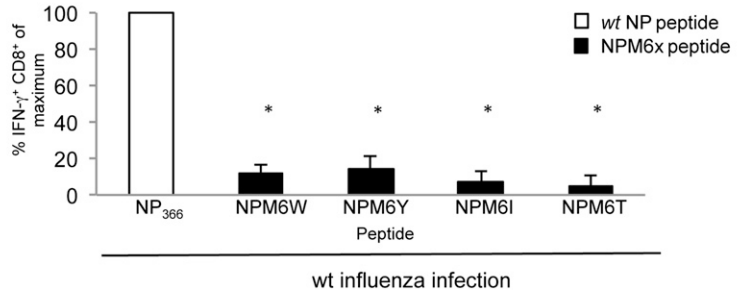
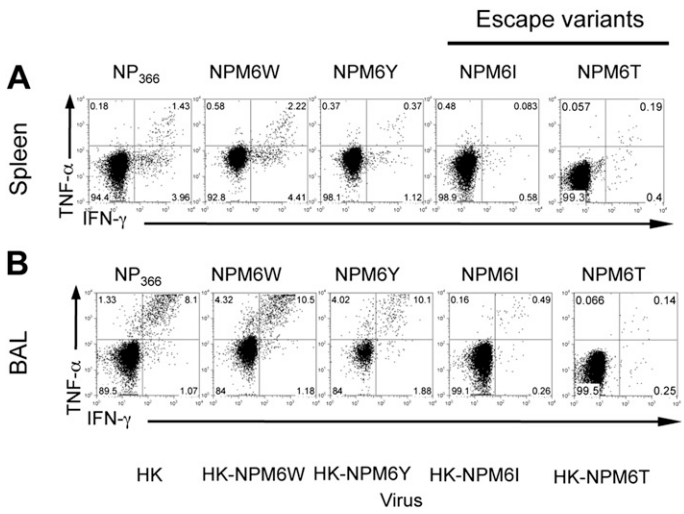


Fig. S1. The *wt* D^{b}NP-specific CD8$^+$ T cells show little cross-reactivity with the substituted NPM6X peptides. (*A*) Various amino acid substitutions at PM6 for the NP$_{366}$ peptide caused biochemical changes that in no way diminished the thermostability of NPM6X binding to H-2D^b. Temperatures (°C) represent the point where 50% of the peptide-MHC was unfolded. ^Amino acid substitutions were either conserved or nonconserved. The NPM6I and NPM6T escape variants were previously isolated from the lungs of infected mice (1). (*B*) Spleen cytotoxic T lymphocytes (CTLs) from mice primed intraperitoneally with the *wt* PR8 virus, then challenged intranasally with the *wt* HK virus 6 wk later, were analyzed for CD8$^+$ T-cell specificity on day 8 after secondary infection. Cells were stimulated directly ex vivo for 5 h (in the presence of Brefeldin A) with either the *wt* NP$_{366}$ or a panel of NPM6X peptides. IFN-γ production was measured by intracellular cytokine staining (ICS), and the background fluorescence was subtracted for the "no peptide" controls. Data represent mean± SD (n = 5). Experiments were repeated at least twice. *$P \leq 0.01$ relative to *wt* HK NP$_{366}$-specific response.

1. Wu TD, Brutlag DL (1996) Discovering empirically conserved amino acid substitution groups in databases of protein families. *Proc Int Conf Intell Syst Mol Biol* 4:230–240.
2. French S, Robson B (1983) What is a conservative substitution? *J Mol Evol* 19:171–175.
3. Price GE, Ou R, Jiang H, Huang L, Moskophidis D (2000) Viral escape by selection of cytotoxic T cell-resistant variants in influenza A virus pneumonia. *J Exp Med* 191(11):1853–1867.



C Spleen

Virus	Peptide	# Mean	SD	P values
HK	NP	323908.45	71064.50	
HK-NPM6I	M6I	39238.75	32779.61	>0.0001
HK-NPM6T	M6T	23065.25	15432.12	>0.0001
HK-NPM6W	M6W	472316.95	134165.47	0.06(above wt)
HK-NPM6Y	M6Y	128990.28	70575.50	0.002

D BAL

Virus	Peptide	# Mean	SD	
HK	NP	9557.1	1982	
HK-NPM6I	M6I	1671.03	1024.86	>0.0001
HK-NPM6T	M6T	2249.02	782.48	>0.0001
HK-NPM6W	M6W	20150.06	18318.86	NS
HK-NPM6Y	M6Y	6675.18	4401.63	NS

Fig. S2. The NPM6I and NPM6T escape variants generate minimal $CD8^+$ T-cell responses. Following intranasal infection with either the WT HK or mutant HK-NPM6X virus, (*A* and *C*) splenocytes or (*B* and *D*) BAL cells (from the site of infection) were stimulated with the WT NP or NPM6X peptides corresponding to the infecting virus. (*A* and *B*) Representative FACS plots are shown. (*C* and *D*) Numbers of epitope-specific $CD8^+$ T cells in (*C*) spleen and (*D*) BAL are presented as mean ± SD. Background "no peptide" values were subtracted. Experiments were repeated at least twice. *P* values are shown for the comparison between the WT $D^bNP_{366}{}^+CD8^+$ T-cell responses and $NPM6X^+CD8^+$ populations.

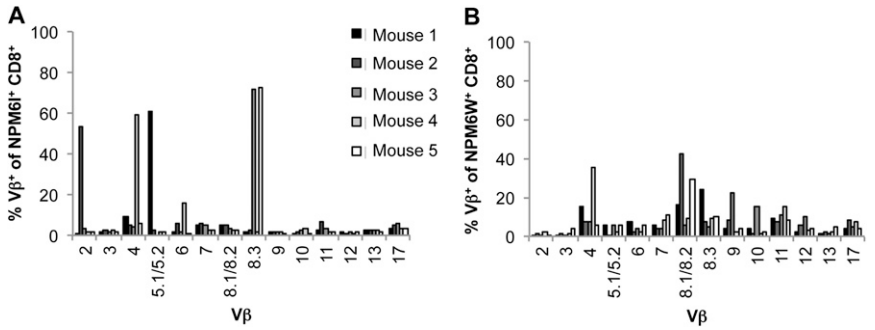


Fig. S3. Limited T-cell receptor (TCR) Vβ use for the low precursor frequency $NPM6I^+$ $CD8^+$ T cells. Splenocytes were stained with D^bNPM6X-allophycocyanin tetramer and a panel of 14 FITC mAb specific for the TCR Vβ region. Vβ use was assessed for secondary acute responses generated by homologous priming with PR8-NPM6I followed by infection with (*A*) HK-NPM6I or (*B*) HK-NPM6W. Data represent individual responses (n = 4–5 mice).

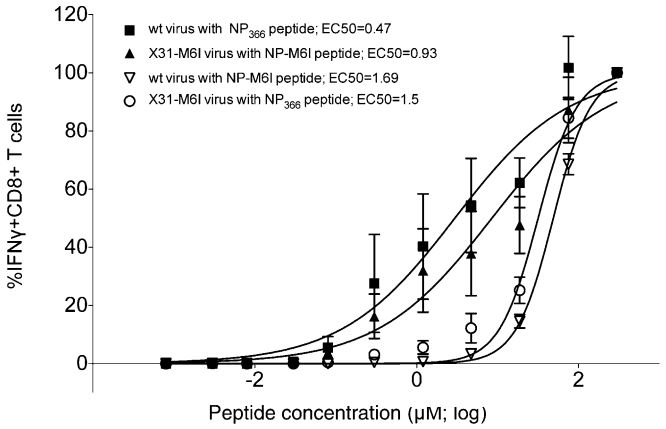


Fig. S4. Optimal stimulation with the homologous peptides. Responsiveness of $CD8^+$ T cells to limited peptide concentrations was determined as a measure of functional TCR avidity for the cognate peptide and class I MHC (pMHCI) complex. $D^bNP_{366}{}^+CD8^+$ and D^bNPM6I $CD8^+$ T cells recovered from mice primed and challenged with the *wt* or NPM6I viruses (PR8 prime, HK boost) were assessed for functional TCR avidity using the IFN-γ ICS assay. The enriched splenoctytes analyzed by IFN-γ ICS were stimulated in vitro with fourfold dilutions of the NP_{366}, or NPM6I peptide. Data represent mean± SD (n = 4) and were plotted as sigmoidal curves by transforming peptide concentrations to log values. EC_{50} values are shown in the figure.

Table S1. Data collection and refinement statistics

Data collection and refinement	Db-NPM6T	Db-NPM6W	Db-NPM6I	Db-H155A-NP	Db-H155AA-NPM6I
Data collection statistics					
Temperature	100K	100K	100K	100K	100K
Space group	P 2_1	P 2_1 2_1 2	P2	C2	P2
Cell dimensions	79.72, 84.40, 136.03	86.28, 153.62, 80.50	83.69, 72.27, 86.83	92.33, 111.39, 58.00	83.95, 72.93, 86.35
(a, b, c), Å	β = 90.89°		β = 103.28°	β = 122.72°	β = 103.10°
Resolution, Å	100–2.60 (2.70–2.60)	100–2.50 (2.60–2.50)	50.00–2.00 (2.10–2.00)	100–2.20 (2.30–2.20)	100–2.00 (2.10–2.00)
Total no. of observations	435,398 (42,786)	259,485 (26,613)	284,953 (36,523)	93,158 (11,487)	254,265 (35,169)
No. of unique observations	52,751 (5,093)	35,931 (3,672)	64,509 (8,223)	24,876 (3,089)	68,339 (9,332)
Multiplicity	8.2 (8.4)	7.2 (7.2)	4.4 (4.4)	3.7 (3.7)	3.7 (3.7)
Data completeness, %	94.5 (85.3)	94.0 (88.5)	94.4 (89.1)	98.7 (98.4)	99.3 (99.9)
I/σ_I	15.5 (3.1)	15.9 (3.5)	11.5 (3.2)	12.4 (2.6)	13.5 (2.8)
R_{merge},* %	11.7 (68.8)	10.9 (58.9)	10.3 (48.0)	8.2 (58.5)	6.7 (48.3)
Refinement statistics					
Nonhydrogen atoms					
Protein	12,768	6,322	6,453	3,216	6,403
Water	211	181	848	171	830
No. of monomer	4	2	2	1	2
R_{factor},† %	17.8	20.9	19.1	19.4	18.1
R_{free},† %	24.7	24.5	23.1	23.7	23.6
RMS bond lengths, Å	0.010	0.010	0.009	0.010	0.010
RMS bond angles, °	1.17	1.17	1.06	1.12	1.09
Ramachandran plot, %					
Favored/allowed regions	99.2	98.2	99.1	98.8	99.4
Generously allowed regions	0.7	1.1	0.6	1.2	0.6
Disallowed regions	0.1	0.8	0.3	0	0
PDB code	4HUW	4HUV	4HUU	4HUX	4HV8

Values in parentheses are for the highest-resolution shell.

*$R_{merge} = \Sigma \mid I_{hkl} - < I_{hkl} > \mid / \Sigma I_{hkl}$.

†$R_{factor} = \Sigma_{hkl} \mid \mid F_o \mid - \mid F_c \mid \mid / \Sigma_{hkl} \mid F_o \mid$ for all data except ~ 5% which were used for R_{free} calculation.

Table S2. Frequency of TCRβ clonotypes in $D^bNPM6I^+Vβ8.3^+CD8^+$ cells and D^bNPM6W^+ $CD8^+$ T cells within multiple Vβ, after infection with HK-NPM6I or HK-NPM6W viruses

			Frequency, %				
CDR3β	Jβ	Amino acid length	M1	M2	M3	M4	M5
D^bNPM6I							
Vβ8.3							
SDWRGEQ	2.6	7	95				
SDARGFLAEQ	2.1	10	5				
SDWGTGGKAEQ	2.1	11		100			
Vβ1							
SQDLGGIYEQ	2.7	10			100		
Vβ4							
SQEAGGYEQ	2.7	9				93	
SQDRTGGRDEQ	2.7	11				3	
SQDEFANTEV	1.1	10				3	
Total sequences			40	35	32	30	
D^bNPM6W							
Vβ4							
SQYWGGANEQ	2.1	10				15	
SQDRGLEQ	2.6	8				27	
SQDRRNSYNSPL	1.6	12				29	
SQEGTGYSGNTL	1.3	12				27	
SQYWGGYAEQ	2.1	9				2	
Vβ8.1							
GAGLGGRAETL	2.3	11		6			
GDARTGVAETL	2.3	11					6
GDASLYAEQ	2.1	9		3			
GDHRGGIYAEQ	2.1	11					3
GDRDDNYAEQ	2.1	10		6			
GDTGRYAEQ	2.1	9		6			
GEPGLGPYEQ	2.6	10					6
GGGTFVYEQ	2.6	9		10			
GGLGGNYAEQ	2.1	10					54
GVGSNYAEQ	2.1	9		26			
GVRQSSGNTL	1.3	10					20
Vβ8.2							
SDSWGGAEQ	2.6	9					
SEHRGRTEV	1.1	9		26			
SPGRGGAEE	2.1	9		6			
SPGRGGAEQ	2.1	9		10			
Vβ8.3							
SDGTGAQDTQ	2.5	10	89				
SDGTGAKTPSTLGQ		15	3				
SDGTGAQDTSTLGQ		15	3				
SDRDRVYAEQ	2.1	10	3				
SENGVEQ	2.6	7	3				
Vβ9							
SRIRGGRDTQ	2.5	10			18		
SRDRAEQ	2.6	7			32		
SHRGLNQAP	1.5	9			47		
RHRGANTEV	1.1	9			3		
Total sequences			38	31	34	41	35

Table S3. Summary of TCRβ data for $D^bNP_{366}^+$, D^bNPM6I^+ and D^bNPM6W^+ $CD8^+$ T cells

Summary	D^bNP_{366}^*	D^bNPM6I	D^bNPM6W
Mice analyzed	10	4	4
TCRs sequenced	976	137	179
Vβ population sequenced	8.3	8.3, 1, 4	4, 8.1/8.2, 8.3, 9
Predominant Jβ region	2.2, 1.1	2.6, 2.1, 2.7	1.1, 1.3, 1.5, 2.1, 2.5, 2.6
Predominant CDR3β length	9	7–11	7–12
Repeated (shared) sequence	3	None	None
Clonotypes per mouse (amino acid)	7.9 ± 2.5	1.75 ± 0.95	5.75 ± 2.2
Simpson's Diversity Index	0.52 ± 0.2	0.06 ± 0.07*	0.63 ± 0.25

^*Analysis from Turner et al. (1). $D^bNPM6I^+CD8^+$ TCRβ repertoire was analyzed following the 2° challenge with mutant HK-NPM6I virus; $D^bNPM6W^+CD8^+$ TCRβ repertoire was analyzed following 1° infection with HK-NPM6W virus. M, an individual mouse. *$P > 0.01$ relative to wt.

1. Turner SJ, et al. (2005) Lack of prominent peptide-major histocompatibility complex features limits repertoire diversity in virus-specific CD8+ T cell populations. *Nat Immunol* 6(4): 382–389.