HLA class I molecules consistently present internal influenza epitopes

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Cytotoxic T lymphocytes (CTL) limit influenza virus replication and prevent morbidity and mortality upon recognition of HLA class I presented epitopes on the surface of virus infected cells, yet the number and origin of the viral epitopes that decorate the infected cell are unknown. To understand the presentation of influenza virus ligands by human MHC class I molecules, HLA-B*0702-presented viral peptides were directly identified following influenza infection. After transfection with soluble class I molecules, peptide ligands unique to infected cells were eluted from isolated MHC molecules and identified by comparative mass spectrometry (MS). Then CTL were gathered following infection with influenza and viral peptides were tested for immune recognition. We found that the class I molecule B*0702 presents 3–6 viral ligands following infection with different strains of influenza. Peptide ligands derived from the internal viral nucleoprotein ($NP_{418-426}$ and $NP_{473-481}$) and from the internal viral polymerase subunit PB1 ($PB1_{329-337}$) were presented by B*0702 following infection with each of 3 different influenza strains; ligands $NP_{418-426}$, $NP_{473-481}$, and $PB1_{329-337}$ derived from internal viral proteins were consistently revealed by class I HLA. In contrast, ligands derived from hemagglutinin (HA) and matrix protein (M1) were presented intermittently on a strain-by-strain basis. When tested for immune recognition, HLA-B*0702 transgenic mice responded to $NP_{418-426}$ and $PB1_{329-337}$ consistently and $NP_{473-481}$ intermittently while ligands from HA and M1 were not recognized. These data demonstrate an emerging pattern whereby class I HLA reveal a handful of internal viral ligands and whereby CTL recognize consistently presented influenza ligands.

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Cytotoxic T lymphocytes (CTL) kill influenza-infected cells upon recognition of distinct class I HLA peptide complexes at the cell surface. Class I molecules sample the proteome of the infected cell and display on the cell surface short viral peptides 8–12 aa in length to surveying immune cells (1). Two factors have complicated our understanding of the class I HLA-presented epitopes that distinguish influenza-infected cells. First, the HLA class I peptides of influenza-infected cells have not been directly characterized to assess the nature and breadth of viral peptides that are available for review by CTL. Our laboratory has pioneered the direct elution of such endogenously processed viral ligands and as such is positioned to identify and characterize class I HLA viral peptides of infected cells.

A second factor that complicates the characterization of influenza immune epitopes is virus variability. It is well documented that antibodies which recognize the HA and NA molecules of one influenza strain may not sufficiently bind to HA and NA molecules of older and/or future strains (2–4). Viral peptides presented by class I HLA and targeted by CTL likewise exhibit variability through the emergence of viral escape mutants (5, 6). Although our knowledge of class I-presented influenza epitopes is incomplete, extensive variability has been reported in some of the viral sequences so far reported as CTL targets (5–7). Understanding the nature and number of viral epitopes presented following infection would provide important insights to indicate the epitopes that facilitate virus escape and those epitopes upon which the immune response recognizes despite virus variability.

As a foil to virus variability, humans are able to present peptide epitopes using a diverse array of HLA class I molecules. For viruses such as HIV and EBV it has been observed that class I molecules encoded at the HLA-B locus contribute heavily to anti-viral CTL immune responses, and HLA-B molecules appear key to influenza CTL immunity as well. For example, HLA-B*2705 and -B*3501 represent primary targets for influenza-specific CTL responses in comparison to HLA-A*0201 and -A*0101 restricted CTL responses (8). In addition, influenza epitopes that elicit IFN-γ or cytotoxic responses have been described for 7 HLA-B alleles (B*0702, B*08, B*14, B*27, B*3501, B*37, and B*44) as compared to only 5 HLA-A alleles with an average of 2.1 and 1.4 epitopes presented per HLA-B and HLA-A allele, respectively (9). HLA-B molecules therefore play an integral role in directing CTL responses to a number of viruses including HIV (10, 11), EBV (11, 12), and influenza (8, 13).

The ideal influenza vaccine would generate CTL that recognize highly conserved viral peptides presented by the class I of infected cells. However, there is considerable uncertainty as to the number and nature of viral epitopes presented by class I HLA following infection. In addition, it is unclear how strain-to-strain variability will impact epitope presentation. In this study we characterized the repertoire of viral ligands presented by HLA-B*0702 during infection with three different influenza A strains: A/Puerto Rico/8/34 (PR8) (H1N1), A/Oklahoma/7485/01 (7485) (H1N1), and A/Oklahoma/309/06 (309) (H3N2). Class I HLA/peptide complexes were purified from uninfected and influenza-infected cells and eluted peptides were separated and mapped by reverse-phase HPLC and mass spectrometry (MS). Mass spectrometric analysis identified multiple viral ligands unique to HLA-B*0702 of infected cells. Particular viral peptides were intermittently presented depending upon the viral strain while other ligands were consistently revealed despite sequence variation between strains. Most intriguing was that CTL from infected mice focused upon the "perpetual" influenza epitopes and not the intermittent targets. Understanding the number and nature of these perpetual influenza epitopes in the context of influenza immunity is discussed.

Results

Direct Discovery of Naturally Processed HLA-B*0702 Influenza Peptides. The primary objective of this study was to ascertain the breadth and source of viral ligands presented by HLA-B*0702 during infection with laboratory and circulating strains of influenza A virus. We characterized B*0702 as it is the most common HLA-B allele in the North American population (28% U.S. Caucasian and

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16% African American populations) (14). First we characterized endogenously loaded peptides following infection of cells by influenza PR8, a well-characterized H1N1 laboratory strain. Following analysis of HLA-B*0702 peptides during PR8 infection, we turned to ligands encoded by influenza A H1N1 and H3N2 isolates 7485 (A/New Caledonia/20/99-like) and 309 (A/Wisconsin/67/2005-like), respectively; vaccine-like strains of influenza. We applied our method that utilizes secreted class I molecules to obtain HLA B*0702-peptide complexes from naïve and influenza virus infected HeLa cells. We used intracellular staining with an antibody directed against nucleoprotein to confirm that the cells producing HLA were >50% influenza infected. Using this approach ≈25 mg of sHLA B*0702 peptide complex were harvested and affinity purified from ≈7.5×10^9 naïve and infected cells. From this, ≈500 μg of peptide were obtained from the HLA-B*0702 of both infected and uninfected cells. Ten percent of the infected/uninfected peptide pools underwent Edman degradation to demonstrate that peptides were eluted from B*0702 (data not shown) and the remaining infected/uninfected peptides were comparatively analyzed and mapped by RP-HPLC and mass spectrometry to identify ligands unique to infected cells.

Comparative Mass Spectrometry Reveals 7 Influenza HLA B*0702 Ligands. Comparative RP-HPLC (Fig. 1*A*) and mass spectrometric mapping identified ions (putative peptides) found only in influenza-infected cells (Fig. 1*B*). Ions characteristic to the MS maps of infected cells were subjected to MS/MS fragmentation to determine the amino acid sequence of these peptide ligands unique to infected cells (Fig. 1*C*). A total of 7 HLA-B*0702 influenza ligands were identified by comparative MS ion mapping and MS/MS sequencing following infection with the 3 different viral strains. For each viral strain, 3–6 viral ligands were presented by B*0702 (Fig. 2). Noteworthy is that the majority of B*0702-presented influenza peptide ligands derive from internal viral proteins: Peptides from the internal nucleoprotein (NP), polymerase basic protein 1 (PB1), and matrix protein (M1) were identified. The lone exception was the hemagglutinin$_{339-347}$ (HA) peptide IPSIQSRGL that was presented only during infection with PR8. Thus, HLA-B*0702 sampled predominantly internal viral proteins.

Among the 7 influenza peptides discovered by this technique, 4 (NP-, PB1-, and HA-derived peptides) were nonamers with the characteristic HLA B*0702 binding motif of a proline at P2 and leucine or methionine at position 9, the C terminus. Three M1 peptides varied in length (7-mer, 8-mer, and 10-mer) and lacked the conventional HLA-B*0702 P2 and C-terminal anchor residues. A synthetic competitive binding assay revealed that while the HA, PB1, and NP peptides had a high or medium affinity for B*0702 (high affinity: $\log(IC_{50}\ nM) \leq 3.7$; medium affinity: $\log(IC_{50}\ nM) \leq 4.7$), the M1 peptides had a very low affinity for B*0702 (Table 1). The low binding affinity of M1 peptides is likely because of their size and/or lack of the 2 characteristic B*0702 anchor residues. This is consistent with reports of other influenza matrix ligands that lack a characteristic class I anchor residue and exhibit a low binding affinity (15). With the exception of the matrix peptides, the ligands reported here are consistent in size, binding affinity, and sequence with previously reported B*0702 epitopes of viral and human origin (9).

$NP_{418-426}$ Is Consistently Presented by HLA B*0702 Despite Strain-to-Strain Variability in This Region of the NP molecule. HLA-B*0702 presents a peptide derived from amino acid positions 418–426 of the nucleoprotein molecule for all 3 strains of influenza tested (Fig. 2). Presentation of $NP_{418-426}$ occurred despite substantial variability at peptide positions 4–8 for the 3 strains tested (Table S1). While variability was observed at positions 4–8 of the $NP_{418-426}$ ligand, the characteristic B*0702-preferred P2 proline and the C-terminal methionine showed no strain-to-strain variability. Collectively, these data document that extensive $NP_{418-426}$ variability is sandwiched between a conserved P2 proline and a conserved C-terminal methionine such that $NP_{418-426}$ is consistently sampled by HLA-B*0702.

In addition to $NP_{418-426}$, B*0702 sampled $NP_{473-481}$ (SPIVPSFDM-PR8 and NPIVPSFDM-7485/309) and $PB1_{329-337}$ (QPEWFRNVL-PR8 and QPEWFRNIL-7485/309) in all of the influenza strains tested (Fig. 2). The $NP_{473-481}$ ligand is moderately conserved among influenza H1N1 and H3N2 strains; the consensus sequence NPIVPSFDM exhibits 77% conservation among different viral strains (16). Thus, 2 NP peptides were consistently presented: one of which is presented despite hypervariability within the ligand and the second of which is relatively conserved among different strains of influenza. Like the $NP_{473-481}$ ligand, $PB1_{329-337}$ is fairly well conserved across H1N1 and H3N2 influenza virus strains; the $PB1_{329-337}$ sequence QPEWFRNIL is conserved among 93% of reported H1N1 and H3N2 strains (16).

These results indicate that only a handful of viral ligands derived primarily from internal viral proteins are presented during influenza infection by the most common HLA-B molecule in the North American population. Three peptides ($NP_{418-426}$ epitope, $NP_{473-481}$, and $PB1_{329-337}$) were presented by B*0702 for all infecting strains of influenza while other peptides were presented on a strain-by-strain basis. Most noteworthy was the B*0702 presentation of the $NP_{418-426}$ ligand despite substantial strain-to-strain variability in the center of this peptide.

HLA-B7 Supertype Family Member HLA-B*3501 Exhibits Variable Presentation of HLA-B*0702 Influenza Peptides. To this point we have fixed the HLA-B molecule and varied the viral strain. Here we test the peptides presented by other class I HLA molecules. The HLA-B7 supertype is a grouping of HLA-B class I molecules based upon their affinity for binding peptides with a proline at the P2 anchor position (17). Class I HLA-A and -B alleles can be grouped together into HLA supertype families on the basis of the amino acids preferred at peptide anchor positions. There are currently 6 HLA-A and 6 HLA-B class I supertype families (17). We therefore examined the presentation of the B*0702 NP and PB1 ligands by another member of the B7 supertype. Peptides characteristic of HLA-B*3501, a member of the HLA-B7 supertype, exhibit proline and tyrosine at the P2 and C-terminal anchor positions, respectively. HLA-B*3501 is the second most common allele within the HLA-B7 supertype, exhibiting a phenotype frequency of 10.6% and 12.4% in U.S. African-American and Caucasian populations, respectively (14). Mass spectrometric analysis of sHLA-B*3501 eluted peptides gathered from 309 (H3N2) infected HeLa cells identified nucleoprotein ligands 418–429 (LPFEKSTIM) (Fig. S1) and 473–481 (NPIVPSFDM) but not the PB1 peptide (Fig. 2). These data indicate that the presentation of HLA-B*0702 NP influenza ligands occurs across multiple alleles within the HLA-B7 supertype while other viral peptides were not presented by both HLA-B*0702 and its supertype relative B*3501.

CTL of Influenza PR8 Infected HLA B*0702 Transgenic H-2K^{b}D^b Double-Knockout Mice Recognize Directly Discovered Influenza Epitopes. Having discovered B*0702 ligands unique to, and presented across, different strains of influenza, we next tested the immunogenicity of these viral peptides. HLA-B*0702 transgenic mice were inoculated intranasally with PR8 or endotoxin free saline (mock infection) and then splenocytes from these mice were tested for IFN-γ reactivity to the 7 influenza peptides identified by mass spectrometry via ELISPOT (Fig. S2, Table 2). Two influenza peptides, $NP_{418-426}$ and $PB1_{329-337}$, generated an IFN-γ response in all PR8-infected mice tested, yielding an average of 157 and 193 spot forming units (SFU)/10^5 lymphocytes, respectively (Fig. S2, Table 2). On the basis of SFU and the number of responding mice, $NP_{418-426}$ and $PB1_{329-337}$ were clearly the immunodominant epitopes in response to a viral challenge.

The number of SFU and the number of responding mice were

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Fig. 1. Identification of $NP_{418-426}$ by mass spectrometry during infection with multiple influenza A virus strains. (*A*) Peptides eluted from sHLA-B*0702 of naïve (red) and influenza infected (black) HeLa cells were purified and separated by RP-HPLC. Corresponding RP-HPLC fractions from naïve and infected cells were sprayed via nanospray into a Q-TOF mass spectrometer to create MS ion maps for each fraction. (*B*) MS ion maps of RP-HPLC fraction 63 from infected (upper panel) and naïve (lower panel) cells were compared to identify ions (putative peptides) unique to infected cells. (*C*) The hypervariable NP 418–426 ligand was identified during infection of sHLA-B*0702 HeLa cells with PR8 (*Upper*), 7485 (middle panel), and 309 (lower panel) influenza strains. MS/MS fragmentation creates a series of b and y ions as the peptide is fragmented from the N (b ions) and C (y ions) terminus which are used to determine the peptide's amino acid sequence.

noticeably lower for other viral peptides. Five of 8 PR8 infected mice produced IFN-γ in response to $NP_{473-481}$, with an average SFU of 13.59. The directly discovered $HA_{339-347}$, $M1_{200-206}$, $M1_{199-206}$, and $M1_{229-238}$ ligands identified here were negative by ELISPOT in this animal challenge model.

Both CD8 and CD4 T cells contribute to the anti-viral IFN-γ response during influenza infection (18–20). The HLA-B*0702 transgenic mice used in this study are on the C57BL/6 background and express the I-A^b class II MHC allele. The class II molecule I-A^b binds peptide ligands with tyrosine (Y) or phenylalanine (F) at the

Fig. 2. HLA-B*0702 and HLA-B*3501 directly identified influenza A virus peptides. Peptides eluted from sHLA of HeLa cells infected with either PR8 (H1N1), 7485 (H1N1), or 309 (H3N2) strains were analyzed by mass spectrometry to identify viral peptides. During influenza A infection HLA-B*0702 presented 3–6 viral ligands. Three viral peptides (NP 418–426, NP 473–481, and PB1 329–337) were eluted from the sHLA of HeLa cells infected with all three influenza A virus strains. Ligands derived from HA and M1 were HLA-B*0702 presented during infection with one influenza A strain. Both NP 418–426 and NP 473–481 were presented by HLA-B*3501, another allele within the HLA-B7 supertype.

first anchor position (i) and uncharged residues at positions $i + 5$ and $i + 8$ (21). Of the 7 directly discovered influenza A virus ligands, only the $NP_{418-426}$ peptide contains the I-A^b binding motif. However, it is unlikely that the $NP_{418-426}$ IFN-γ response generated by lymphocytes isolated from PR8 infected HLA-B*0702 transgenic mice is largely because of CD4 T cell reactivity given that the $NP_{418-426}$ epitope elicits CD8 T cell reactivity during in vitro stimulation of human peripheral blood mononuclear cells (PBMC) isolated from healthy HLA-B*0702 and -B*3501 adults (13, 22–24).

Discussion

Studies of humoral immunity indicate that influenza evades antibody responses by varying targeted epitopes while a flexible immune system counters by generating responses to a number of different viral sequences. While humoral responses prevent infections (3), HLA class I mediated CTL responses are integral to the clearance of influenza infections (25–27). To provide an understanding for how class I HLA expose influenza epitopes to host CTL, the goals of this study were to (i) provide an understanding of how many viral ligands are revealed by an individual class I HLA molecule, (ii) characterize the impact that strain-to-strain variability has upon class I HLA epitope presentation, and (iii) assess the immunogenic nature of the viral peptides revealed. Realizing how a class I molecule makes an influenza infection known to the host, and how virus strain-to-strain variability impacts HLA exposure, is key to orchestrating protective viral immunity. A priori, it was possible that we would find a vast array of peptide epitopes derived from all 8 virally encoded sequences of the influenza genome; the other extreme would be that 1 or only a few virally encoded sequences were presented by HLA molecules to the CTL scanning system of the host.

The most important finding of this study is that, by direct MS characterization of peptides eluted from sHLA-B*0702 during influenza infection, only 3–6 viral peptides are presented. We arrived at this important conclusion after infecting cells with 3 different strains of influenza A and testing 2 different HLA class I molecules. While we cannot say that all class I molecules will sample a like number of viral peptides, these data are consistent with recent data published from our laboratory in another human viral system, West Nile Virus (WNV), where we showed that only 6 ligands were presented by HLA-A*0201 (28). Thus, data with 2 different viruses (WNV and influenza) and 2 different class I molecules (A*0201 for WNV and B*0702 for influenza) provide evidence that, when a class I molecule samples viral peptides, only a modest number (3–6) of viral ligands are presented to CTL for immune recognition.

Table 1. Naturally processed influenza A HLA B*0702 class I peptides

HPLC fraction	AMU	Influenza strain	Protein	Peptide sequence	Log [IC50 nM]
55	485.8	PR8	HA 339–347	IPSIQSRGL	2.221
47	720.3	7485	M1 200–206	AEAMEVA	>6
47	791.4	7485	M1 199–206	AAEAMEVA	>6
62	600.3	7485	M1 229–238	LKNDLLENLQ	5.695
61	540.3	PR8	NP 418–426	LPFDRTTVM	1.85
63	533.3	7485	NP 418–426	LPFDKTTIM	2.284
63	533.3	309	NP 418–426	LPFEKSTIM	3.892
68	496.8	PR8	NP 473–481	SPIVPSFDM	2.551
66	510.2	7485/309	NP 473–481	NPIVPSFDM	3.09
71	594.8	PR8	PB1 418–426	QPEWFRNVL	2.592
73	601.8	7485/309	PB1 418–426	QPEWFRNIL	2.275

AMU, atomic mass unit.

Table 2. Influenza B*0702 peptides tested for ELISPOT reactivity in 8 PR8-infected HLA-B*0702 transgenic mice

Peptide Sequence	Source Protein	Average SFU/10^5 cells	Mice Positive
LPFDRTTVM	NP 418–426	157	8/8
QPEWFRNVL	PB1 329–337	193.08	8/8
SPIVPSFDM	NP 473–481	13.59	5/8
IPSIQSRGL	HA 229–347	0.67	0/8
AEAMEVA	M1 200–206	0.71	0/8
AAEAMEVA	M1 199–206	0.75	0/8
LKNDLLENLQ	M1 229–238	0.59	0/8

A second important finding of our study was the internal nature of the viral proteins sampled. With the exception of HA, the NP, PB1, and M1 peptides are all derived from internal viral proteins. There has been speculation that viral ligands presented by HLA class I during influenza infection are derived from internal proteins, as influenza-specific CTL described in the literature are often directed against internal viral proteins. The majority (27) of known influenza CTL epitopes are derived from the NP molecule (9). The sampling of *internal* viral proteins by class I HLA is an elegant complement to humoral responses that target the solvent-accessible epitopes of *external* viral proteins: In combination, the humoral and cellular components of the adaptive immune system target epitopes from both internal and external viral proteins. The observation that internal viral proteins are more conserved than surface proteins (29) suggests that internal proteins may be more promising immunotherapeutic agents. However, such a conclusion is premature as we cannot predict whether various HLA will interact with influenza in a fashion similar to the common molecule HLA-B*0702. Experiments are underway to test this possibility.

Virus variability is positioned to thwart successful immune recognition, and the characterization of B*0702 ligand sampling with 3 different influenza A strains allowed us to assess consistency in viral antigen presentation. We found that 3 epitopes, $NP_{473-481}$, $NP_{418-426}$, and $PB1_{339-347}$, are presented during infection with the 3 influenza A strains tested. Moreover, vigorous immune responses are mounted to 2 of these 3 consistently presented epitopes; all mice challenged with influenza exhibited an average of >150 SFU to $NP_{418-426}$ and $PB1_{339-347}$. $NP_{418-426}$ and $PB1_{339-347}$ are therefore immunodominant B*0702 epitopes. Immunodominance of 1–2 influenza CTL epitopes has been demonstrated for multiple class I HLA alleles including HLA-A*0201 (30–32). In follow-up experiments, B*0702 epitopes are presented by B*3501, another member of the B7 supertype. We found that B*3501 presents the 2 NP epitopes but not the PB1 epitope. These data show that multiple viral ligands are available for immune inspection and that $NP_{418-426}$ emerges as an immunodominant epitope presented by multiple members of the B7 supertype.

The $NP_{418-426}$ epitope has been characterized as hypervariable and therefore has been considered an unlikely CTL epitope (33). Among all reported human influenza A H1N1 and H3N2 strains, 21 different $NP_{418-426}$ amino acid sequence variants exist (Table S1). The most likely explanation as to why $NP_{418-426}$ is consistently presented is that HLA-B*0702 binds ligands via a P2 proline, and studies demonstrate that amino acids other than proline at this position in NP severely reduce viral fitness (34). Unlike the $NP_{418-426}$ epitope, the amino acid sequence of the $NP_{473-481}$ and $PB1_{339-347}$ ligands is fairly conserved among H1N1 and H3N2 isolates. Taken together, these data indicate that $NP_{418-426}$ is an epitope that (*i*) is presented well by the high frequency class I molecule HLA-B*0702 in multiple strains of influenza, (*ii*) is well recognized by CTL, (*iii*) is presented by multiple members of the B7 supertype, and (*iv*) exhibits great diversity in positions 4–8. It therefore appears that members of the B7 supertype have evolved a structure that presents an epitope with a conserved P2 proline anchor and that the influenza virus varies positions 4–8 of this epitope to escape consistent CTL recognition (6).

In agreement with the data presented here, another immunogenicity study using synthetic peptides, HLA-B*0702 transgenic mice, and a PR8 challenge found that CTL recognize $NP_{418-426}$. A synthetic version of the second NP peptide discovered here ($NP_{473-481}$ SPIVPSFDM) tested negative for a cytotoxic T cell response (23). Our data clarify that both NP epitopes are presented following infection yet only $NP_{418-426}$ is recognized by CTL. Synthetic versions of the PB1, HA, and the 3 M1 HLA-B*0702 peptides identified here have not been tested for immune recognition in other studies. Also consistent with our direct epitope discovery and supertype data, $NP_{418-426}$ has been identified as a HLA-B*3501 CTL epitope during human influenza infection (6, 24). The immunogenicity testing of $NP_{418-426}$ with HLA-B*0702 and −B*3501 by others is consistent with our direct discovery approach showing that $NP_{418-426}$ is presented by both members of the B7 supertype and recognized by CTL following infection. Our data demonstrating that influenza strain-to-strain hypervariability does not abrogate $NP_{418-426}$ antigen presentation further highlights the immunodominant nature and potential therapeutic utility of this epitope. Lastly, we identify previously unreported HLA-B*0702 viral ligands, including the $PB1_{339-347}$ epitope that was presented during infection with multiple influenza A strains. Our data suggest that $PB1_{339-347}$ will be highly immunogenic in future studies of influenza infected B*0702 individuals.

In summary, we provide the first direct and systematic characterization of influenza ligands eluted from human class I molecules. We find that 3–6 peptides derived predominantly from internal viral proteins are presented during infection with different strains of influenza A. For the 2 HLA-B molecules tested here, we see that NP-derived epitopes represent the most accessible targets as they are presented across 3 viral strains by 2 class I molecules. A priori one would not select the hypervariable epitope $NP_{418-426}$ as an optimal immune target, yet this epitope is consistently presented and recognized by CTL. It is interesting that another more conserved NP ligand is consistently presented but not targeted [our data and ref. 23]. Future studies should reveal if other HLA-A and HLA-B molecules also present 3–6 ligands derived from internal viral proteins. For now, a pattern is emerging whereby class I HLA expose only a handful of internal viral ligands and whereby CTL continually recognize consistently presented influenza ligands.

Materials and Methods

Cell Lines and Transfectants. HeLa (ATCC CCL-2) cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. The cytoplasmic and transmembrane domains of HLA-B*0702 and -B*3501 alleles were removed via PCR mutagenesis and the resultant secreted HLA (sHLA) class I cDNA construct cloned into a pcDNA 3.1^{-} expression vector (Invitrogen) and electroporated into HeLa cells. Transfectants sHLA production was measured by sandwich ELISA using anti-W6/32 (35) capture and anti-β_2-microglobulin detection antibodies (36).

Virus Production and Cell Pharm Infection. This study focused on 3 influenza A virus strains: PR8, 7485, and 309. PR8 is a well-characterized H1N1 laboratory strain. While, PR8 is antigenically distinct from influenza A virus strains in current circulation, its biology is similar and T cell responses to PR8 have been extensively studied in the mouse model. Human influenza viruses 7485 and 309 are clinical isolates that represent influenza A virus strains that the human population has recently faced. Therefore, we focused on a well-characterized influenza A virus strain (PR8) for initial peptide discovery followed by recent H1N1 and H3N2 influenza strains as representative viruses for currently circulating strains.

Influenza viruses were grown in Madin–Darby canine kidney (MDCK) cells. MDCK cells were seeded into roller bottles at a density of 20,000 cells/cm^2 and 2 days later washed with PBS and inoculated with virus in 10 mL CaMg PBS at an MOI of 0.05. After incubation for 2 h at 37 °C, DMEM/F12K supplemented with 1% penicillin/streptomycin, 1% nonessential amino acids, and 1% sodium pyruvate were added to the cell culture and virus production monitored daily by HA titration on human red blood cells. Cell supernatant containing virus was spun down at 3,000 rpm for 30 min to remove cell debris and stored at −80 °C in 200-mL aliquots.

For cell pharm infection 7.5×10^9 HeLa cells were pelleted, washed 3 times with CaMg PBS, incubated for 2 h at 37 °C with 2×10^{-4} HA units of virus per cell, and grown in a cell pharm CP2500 hollow fiber bioreactor (Biovest International) in DMEM supplemented with 6% ITS. Cells were monitored daily for glucose consumption and pH. sHLA production was monitored by W6/32 ELISA. The percentage of cells infected with influenza was measured by intracellular staining with anti-serum directed against the influenza core (37) or an anti-nucleoprotein molecule antibody (Meridian Life Science Inc.) and flow cytometric analysis.

Peptide Isolation and Purification. Approximately 25 mg of sHLA-peptide complex was affinity purified from cell pharm supernatant with the W6/32 antibody. Peptide was released from class I heavy and light chains by a 10% acetic acid boil and pooled by passage through a stirred cell ultrafiltration device with a 3-kDa

membrane (Millipore). Fourteen cycles of N-terminal Edman degradation of 10% of the naïve and infected peptide pools demonstrated that the eluted peptides fit the HLA-B*0702 peptide binding motif. Uninfected/infected peptide pools were separated by reverse-phase HPLC with a Jupiter Proteo 4-μm, 90-Å, 150 $\times$ 2-mm microbore column and fractions collected every 0.7 min. The naïve and infected peptide pools were separated by RP-HPLC into 40 peptide-containing fractions of $\approx$200 peptides per fraction (38).

Mass Spectrometric Analysis. Peptides in the naïve and infected RP-HPLC fractions were mapped by MS with each fraction sprayed 3 times via nanospray into a Qstar Elite quadrupole time-of-flight mass spectrometer to create reproducible MS ion maps for the peptides in each HPLC fraction. To detect peptides unique to infected fractions, corresponding uninfected/infected MS ion maps were aligned at 20-amu increments and visually assessed for the presence of ions unique to infected MS spectra (39). Peptides exhibiting a $\geq$1.5-fold increase during infection were identified by summing the intensity values for each ion in the three uninfected/infected MS ion maps and calculating the normalized fold increase of each infected ion over uninfected. Selected ions underwent tandem MS/MS fragmentation and the amino acid sequence determined *de novo* and/or by MASCOT (40). A total of 2,691 ions (unique or increased) were selected for sequencing by tandem mass spectrometry (MS/MS). The amino acid sequence of influenza ligands uncovered by mass spectrometry was validated by creating a synthetic of each peptide, subjecting it to the same MS/MS collision conditions, and subsequently comparing the endogenous and synthetic fragmentation patterns.

Peptide Binding Assay. A HLA-B*0702 PolyScreen kit (Pure Protein) was used to determine peptide IC_{50} values. Briefly, FITC labeled control peptide and sHLA were incubated with each peptide until equilibrium of peptide replacement was reached. The fluorescent polarization of the control peptide as read on an Analyst AD plate reader (Molecular Devices) and a dose–response curve was used to calculate peptide IC_{50} values (41–43). High affinity binders: log(IC50 nM) $<$3.7; Medium affinity binders: log(IC50 nM) 3.7–4.7; Low affinity binders: log(IC_{50} nM) 4.7–5.5; Very low affinity binders: log(IC_{50} nM) $\geq$6.0 (*SI Text*).

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