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Structural and Functional Correlates of Enhanced Antiviral Immunity Generated by Heteroclitic CD8 T Cell Epitopes

Jonathan A. Trujillo,*† Stephanie Gras,‡† Kelly-Anne Twist,‡ Nathan P. Croft,‡ Rudragouda Channappanavar,‡ Jamie Rossjohn,‡†§,*2 Anthony W. Purcell,‡† and Stanley Perlman*†,§,*2

Peptides that bind poorly to MHC class I molecules often elicit low–functional avidity T cell responses. Peptide modification by altering the anchor residue facilitates increased binding affinity and may elicit T cells with increased functional avidity toward the native epitope (“heteroclitic”). This augmented MHC binding is likely to increase the half-life and surface density of the heteroclitic complex, but precisely how this enhanced T cell response occurs in vivo is not known. Furthermore, the ideal heteroclitic epitope will elicit T cell responses that completely cross-react with the native epitope, maximizing protection and minimizing undesirable off-target effects. Such epitopes have been difficult to identify. In this study, using mice infected with a murine coronavirus that encodes epitopes that elicit high (S510, CSLWNGPHL)– and low (S598, RCQIFANI)–functional avidity responses, we show that increased expression of peptide S598 but not S510 generated T cells with enhanced functional avidity. Thus, immune responses can be augmented toward T cell epitopes with low functional avidity by increasing Ag density. We also identified a heteroclitic epitope (RCVIFANI) that elicited a T cell response with nearly complete cross-reactivity with native epitope and demonstrated increased MHC/peptide abundance compared with native S598. Structural and thermal melt analyses indicated that the Q600V substitution enhanced stability of the peptide/MHC complex without greatly altering the antigenic surface, resulting in highly cross-reactive T cell responses. Our data highlight that increased peptide/MHC complex display contributes to heteroclitic epitope efficacy and describe parameters for maximizing immune responses that cross-react with the native epitope. The Journal of Immunology, 2014, 192: 5245–5256.

Pathogen and tumor clearance both require effective T cell responses; therefore, any vaccines designed to enhance immune protection against infectious diseases or cancer should include relevant CD8 or CD4 T cell epitopes (1, 2). However, some subdominant epitopes recognized in infectious settings and from many tumors induce weak, low–functional avidity T cell responses that are neither protective against pathogen exposure nor efficacious in diminishing tumor burden (3–9). Several approaches have been used to enhance the functional avidity of T cell responses to tumors (10), adoptive immunotherapy of high-avidity T cell clones (11, 12), and immunization with optimized peptides, including heteroclitic peptides; the latter, although altered in sequence, result in augmented T cell responses to the native epitope (2, 13, 14).

Heteroclitic CD8 T cell epitopes were initially identified in the context of tumors (13). In most instances, heteroclitic peptides display enhanced binding to the MHC molecule (15, 16), although heteroclitic peptides that augment binding to the TCR have also been identified (e.g., see Ref. 17). Heteroclitic epitopes exhibiting augmented MHC class I (MHC I) binding and potentially greater effective peptide/MHC complex (pMHC) surface density may induce a higher functional avidity T cell response. However, whether increased pMHC levels actually result in enhanced functional avidity has not been established because several in vitro studies showed that low levels of peptide expressed on the surface of APCs induced CD8 T cells with high functional avidity. Conversely, higher levels of pMHC expression resulted in the outgrowth of cells with lower avidity for the pMHC (3). Based on these in vitro observations, weakly immunogenic epitopes, which often result from low-affinity pMHC interactions and subsequently exhibit low pMHC density, would be predicted to induce high–functional avidity responses. The relationship between the level of pMHC on the surface of APCs and the subsequent CD8 T cell response has also been investigated in vivo (18–20). Increased epitope density raised the magnitude of the response but did not affect the functional avidity of the primary immune response. Importantly, none of these in vitro or in vivo studies have

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examined the relationship between pMHCI density and functional avidity of the T cell response elicited toward a weakly immunogenic epitope and its corresponding heteroclitic analog.

One concern with the use of heteroclitic epitopes is that a variable fraction of the response may recognize only the modified and not the native epitope (21). The outgrowth of cells that recognize only the modified epitope is not only futile as a vaccine strategy but raises the possibility that the modified epitope-specific response could also respond to a self-epitope. This could initiate or contribute to the development of autoimmune disease, thus making the use of heteroclitic peptides in clinical settings problematic.

Mice infected with a murine coronavirus, the neurotropic JHM strain of mouse hepatitis virus (JHMV) or its recombinant form (rJ), develop acute encephalitis and acute and chronic demyelinating diseases (22). Of note, JHMV is a member of the same group of coronaviruses as two pathogenic human coronaviruses associated with severe respiratory disease (severe acute respiratory syndrome–coronavirus and Middle East respiratory syndrome–corona virus) (23). The CD8 T cell response to JHMV in C57BL/6 (B6) mice is directed at two epitopes, spanning residues 510–518 and 598–605 of the surface glycoprotein (CSLWNGPHL, S510, H-2Db-restricted and RCQIFANI, S598, H-2Kb-restricted) (24). Epitope S510 is immunodominant, induces a high–functional avidity response, and in some settings undergoes mutation as part of viral evasion of the T cell response (25). Epitope S598 is subdominant and binds with lower affinity to the MHCI molecule than peptide S510 (14). Furthermore, the low–functional avidity response to epitope S598 is unable to protect mice after infection with virus mutated in S510 (26). With the goal of the S598-specific response, we optimized peptide binding to Kb by changing a secondary anchor residue at position three of the epitope from Gin to Tyr to match the consensus motif for Kb binding (RCYIFANI; Q600Y) (14). We showed that the modified peptide enhanced the stability of pMHCI and induced CD8 T cells with higher functional avidity toward the native form of the S598 epitope. However, as in other studies of heteroclitic epitopes, we found that a substantial fraction (~35%) of the responding T cells recognized only the variant Q600Y epitope. For the reasons described above, this level of cross-reactivity would be unsuitable in a clinical setting because part of the T cell response would be ineffectual (i.e., these T cells would recognize only the heteroclitic peptide and would not contribute to a protective immune response) and could potentially recognize a self-epitope.

In this study, we investigated a putative mechanism of action of heteroclitic epitopes, designed around the subdominant JHMV S598 epitope. If heteroclitic epitopes function by increasing the effective epitope density on the surface of APCs, one would predict that increasing surface density of the native epitope by another mechanism would also result in the induction of a high–functional avidity T cell response. To evaluate this possibility, we used a vaccinia virus (VacV)–based experimental system to vary Kb/Q600V abundance on APCs. The results showed that increased pMHCI expression of epitope S598, which induces a low–functional avidity response, but not of epitope S510, which stimulates a high–functional avidity response in rJ-infected mice, resulted in the selection of CD8 T cells with enhanced ability to respond to the epitope. We also addressed the problem of selection of T cells that responded to variant but not native epitope by using the crystal structures of Kb/Q598 and Kb/Q600Y (14) to identify additional mutations at sub-anchor position 3 that were predicted to be heteroclitic. We showed that one heteroclitic S598 analog (Q600V) elicited a T cell response that nearly completely cross-reacted with the native epitope. Consistent with the notion that surface density is a major factor in how epitope functions, levels of both Kb/Q600V and Kb/Q600Y were increased compared with Kb/S598.

Materials and Methods

Mice

Six- to 8-wk-old pathogen-free B6 mice were purchased from the National Cancer Institute (Frederick, MD) and housed in the Animal Care Facility at the University of Iowa. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Iowa.

Vaccinia

rJ was propagated on 17C1-1 cells and titrated on HeLa cells expressing the MHV receptor CEACAM1 (27) as previously described (28). VacV-S510, -S598, -S598Q600V, and -S598Q600W were engineered using complementary oligonucleotides containing the DNA sequence of the identified epitope, an N-terminal methionine, and appropriate restriction enzyme sequences. Oligonucleotides for the S510 construct were previously published (24). Oligonucleotides were annealed and ligated into PSC65 (a VacV shuttle vector with a strong synthetic VacV early/late promoter for expressing the gene of interest, provided by Dr. B. Moss, National Institutes of Health). VacV-S was constructed by inserting a deleted S gene (lacking the transmembrane domain) (24) into pSC65. Mice were infected i.p. with 2 3 10^5 PFU recombinant VacV-, and CD8 T cell responses were measured in the spleen. Recombinant JHMV expressing variant S598 epitopes were engineered as previously described (14). Mutations were introduced using a site-directed mutagenesis kit (QuikChange II Site-Directed Mutagenesis kit; Stratagene, La Jolla, CA) and the following primers: Q600V, forward, 5'-CATCTGTTAGTTAATGATCGCTGC-3', reverse, 5'-CCATTTACAATATGTTGCAAAAT-3'; Q600W, forward, 5'-CATCTGTTAGTTAATGATCGCTGC-3', reverse, 5'-CCATTTACAATATGTTGCAAAAT-3'; Q600F, forward, 5'-CATCTGTTAGTTAATGATCGCTGC-3', reverse, 5'-CCATTTACAATATGTTGCAAAAT-3'; Q600R, forward, 5'-CATCTGTTAGTTAATGATCGCTGC-3', reverse, 5'-CCATTTACAATATGTTGCAAAAT-3'. Underlined nucleotides correspond to the Gin to Val or Phe changes. At least two independent isolates of each recombinant virus were propagated and analyzed.

One step viral growth kinetics

Virus was inoculated onto confluent 17C1-1 cells at a multiplicity of infection of 0.1. Cells were harvested at the indicated time points and total virus (cell-associated and cell-free) was titrated as previously described (14).

Purification of MHC/peptide complexes

DC2.4 cells (1 3 10^6), grown as previously described, were infected with VacV-S598, VacV-S598Q600V, VacV-S598Q600W, VacV-S510, or VacV-S at 5 PFU/cell and incubated for 4 h before snap freezing. DC2.4 cells were disrupted by gentle resuspension in a total of 5 ml lysis buffer (0.5% IGEPAL [Sigma-Aldrich], 50 mM Tris [pH 8], 150 mM NaCl, and protease inhibitors [complete protease inhibitor mixture tablet; Roche Molecular Biochemicals]). DC2.4s were disrupted by gentle resuspension in a total of 5 ml lysis buffer without cryogenic milling. Lysates were incubated withrotation for 1 h at 4˚C and cleared by centrifugation. MHC/peptide complexes were immunopurified using specific mAbs 28-14-8S (anti–H-2D) or Y-3 (anti–H-2K) bound to protein A–Sepharose, as previously described (29). Bound complexes were eluted by acidification with 2% B to 45% B over the course of 20 min, collecting 500 m fractions.

Liquid chromatography–multiple reaction monitoring–mass spectrometry

Following peptide elution, samples were concentrated, treated with tris(2-carboxyethyl)phosphine (5 mM) for 30 min at 60˚C, and immediately
analyzed by mass spectrometry. An AB SCIEX QTRAP 5500 mass spectrometer was used for multiple reaction monitoring (MRM) detection, coupled online to an Eksigent nano LC and Nanoflex cHiPLC manifold (Eksigent). Samples (10 μl) were injected and loaded onto a trap column (200 μm × 0.5 mm ChromXP C18-CL, 3 μm, 120 Å) at a flow rate of 10 μl/min in 98% water, 2% acetonitrile, and 0.1% formic acid for 10 min. For online fractionation of samples onto the mass spectrometer, samples were eluted from the trap column and passed over a cHiPLC analytical column (75 μm × 15 cm ChromXP C18-CL, 3 μm, 120 Å) at 300 nl/min under the following buffer B (98% acetonitrile, 0.1% formic acid in water) gradient conditions: 0–3 min 2–100% B, 3–33 min 10–40% B, 33–36 min 40–80% B, 36–38 min hold at 80% B, 38–39 min 80–2% B, followed by equilibration at 2% B until the end of the run at 48 min. The QTRAP 5500 was operated in MRM mode in unit resolution for the quantification of peptides. For each information-dependent acquisition criterion set to trigger an enhanced product ion scan (10,000 Da/s, rolling collision energy; unit resolution) following any MRM transition >500 counts. Optimal MRM Q1→Q3 transition conditions were designed through analytical synthesis of peptides and are listed in Table I. Data analysis was performed using Analyst v1.5.2 (AB SCIEX). Isotopically labeled S598 (S598*, containing a modified isoleucine: ([13C])6, (15N)1, mass shift of +7 Da; Mimosotopes, Clayton, VIC, Australia) was added to samples prior to analysis to control for losses during processing and to provide absolute quantitation as previously described (30).

Circular dichroism

CD spectra were recorded using an AVIV 410-SF CD spectrometer. Wavelength scans were performed between 190 and 250 nm using a 1-mm path length quartz cuvette at 20°C with a sample concentration of 0.178 mg/ml in 10 mM Tris and 150 mM NaCl (pH 8). For thermal denaturation scans, celllity at 218 nm was monitored between 20 and 90°C in 0.5°C steps. Data were normalized and then fit to a Boltzmann sigmoidal function. The temperature at which 50% of the protein complex was unfolded was determined (melting temperature \(T_m\)).

Preparation of brain-derived leukocytes

Brain-derived mononuclear cells were isolated as previously described (31). Briefly, mice were perfused with PBS and brains were harvested, dispersed using 25-gauge needles, and digested with collagenase D (1 mg/ml; Roche Diagnostics) and DNase I (0.1 mg/ml; Roche Diagnostics) at 37°C for 30 min. Mononuclear cells were isolated by passing homogenized tissue through a 70-μm cell strainer, followed by centrifugation through a 30% Percoll gradient (Pharmacia, Uppsala, Sweden).

Intracellular cytokine staining and functional avidity analysis

Mononuclear cells were harvested from the brains or spleens of mice at 7 d postinfection (p.i.) and analyzed for expression of IFN-γ by an intracellular cytokine assay as previously described (32). Briefly, brain-derived cells were stimulated in the presence of APCs (CHB3 cells, B cell line, I-Ab, H-2Db, H-2Kb) pulsed with the indicated peptide for 6 h with 5 μl/ml GolgiPlug (BD Pharmingen). Splenocytes were similarly exposed to peptides, but in the absence of exogenous APCs. Peptides were used at a final concentration of 1 μM, unless indicated otherwise. Cells were stained with Abs specific for CD8 (53-6-7; BD Pharmingen), CD4 (RM 4-5; BD Pharmingen), CD16/CD32 (2.4G2; BD Pharmingen), and IFN-γ (XMG1.2; eBioscience) and analyzed by flow cytometry using a FACSCalibur or a FACSVerso (BD Biosciences, Mountain View, CA). Data sets were analyzed using FlowJo software (Tree Star, Ashland, OR). To assess functional avidity, splenocytes or brain-derived mononuclear cells isolated at day 7 p.i., were stimulated with graded doses of the relevant peptide and examined for INF-γ production. The frequency of CD8 T cells producing IFN-γ at each concentration of peptide was measured and expressed as a percentage of the maximum response detected. Data were fit to sigmoidal dose–response curves and used to calculate the amount of peptide needed to reach a half-maximum response (EC50).

Immunization with peptide-coated dendritic cells

Splenic dendritic cells (DCs) were isolated after s.c. injection of B6 mice with 5 × 10^6 B16 cells expressing H\(\beta\)L (provided by M. Prlic and M. Bevan, University of Washington), pulsed with the indicated peptides at 1 μM. In some experiments (informational concentration), and injected i.v. into mice as previously described (33). Mice were sacrificed 6 d later and epitope S598–specific T cell responses were measured in the spleen as described above.
CD8 T cells were detected using cytokine expression because Kβ/Dβ response to ex vivo stimulation with S598 peptide. S598-specific measuring the percentage of CD8 T cells producing IFN-γ the magnitude of the response in the spleen at day 7 p.i. by infected B6 mice with either VacV-S or VacV-S598 and assessed S598-specific CD8 T cell response induced by low (VacV-S) susceptible to efficient infection with rJ.

To assess the functional avidity of the resultant S598-specific T cell populations, splenocytes from mice infected with VacV-S or VacV-S598 were stimulated with graded doses of S598 peptide and examined for IFN-γ production (Fig. 2A–C). We assessed relative functional avidity by determining the peptide concentration needed to reach a half-maximum response (EC50). Infection with VacV-S induced a low–functional avidity S598-specific CD8 T cell response, similar to that induced by S598 in the rJ-infected brain (14) or rJ in the spleen after i.p. inoculation (Fig. 2D). Remarkably, however, infection with VacV-S598 gave rise to a population of T cells with significantly greater sensitivity to S598, requiring ~1000-fold less peptide to achieve a half-maximum response (Fig. 2B, 2C). Thus, increased abundance of the Kβ/Dβ S598 complex resulted in the greater accumulation of CD8 T cells with high functional avidity. An alternative explanation for these results is that peptide processing was different when S598 was expressed from a minigene versus intact S protein. However, minimal CD8 T cell responses to peptides with N- or C-terminal extensions or partial overlap with S598 (RCQIFANIL, RCQIFANIL, DRCQIFANIL, QIFANIL, QIFANIL) were detectable after immunization with VacV-S (data not shown) and none of these extended forms could be detected by liquid chromatography–tandem mass spectrometry (data not shown), together suggesting that the same formulation of epitope S598 was recognized after immunization with either VacV construct.

During JHMV infection, the immunodominant epitope S510 generates a high–functional avidity CD8 T cell response (24, 43). To determine whether increasing the levels of Dβ/S510 would also enhance the functional avidity of this already potent response, mice were infected with VacV-S or VacV-S510. We observed similar frequencies of CD8 T cells specific for S510 in mice infected with either virus (Fig. 2E). In sharp contrast to S598, equivalently high–functional avidity S510-specific CD8 T cell responses were generated in response to infection with either VacV-S (low Dβ/S510 expression) or VacV-S510 (high Dβ/S510 expression) (Fig. 2F, 2G).

To address whether the functional avidity of the virus-specific response was broadly altered by infection with VacV expressing the S protein versus a minigene construct, we examined the CD8 T cell response to VacV epitope B8(20–27). The percentages of CD8 T cells specific for B8(20–27) were similar in the spleens of mice infected with VacV-S, VacV-S598, or VacV-S510 (Fig. 2H). Additionally, each virus construct induced high–functional avidity B8(20–27)-specific CD8 T cell responses, with equivalent EC50 values (Fig. 2I, 2J). Taken together, these results suggest that the observed differences in the functional avidity of the T cell responses to S598 are most likely the consequence of different levels of cognate Ag.

**Heteroclitic S598 analogs induce higher functional avidity S598-specific CD8 T responses**

We previously showed that whereas the heteroclitic Q600Y peptide elicited a higher functional avidity S598-specific CD8 T cell response than did the native S598 epitope, only a fraction of Q600Y-primed cells recognized the native epitope (14). An ideal heteroclitic analog would increase the number of high-affinity virus-specific effectors while minimizing the expansion of cells that fail to recognize the native viral epitope. To try to achieve this, we selected four additional amino acid substitutions (Val, Phe, Glu, Pro) at position three of the S598 epitope that were predicted to be heteroclitic based on our previous work (14). These modifications were chosen because they are nonhomologous, bulky substitutions, similar to the Gln to Tyr change in our original study. Gln has a polar uncharged side chain, Val, Phe, and Pro, similar to Tyr, contain hydrophobic side chains, and Glu is a charged homolog of Gln. One of these variants (Q600E, RCEIFANIL) was not immunogenic when examined in preliminary experiments (data not shown). We engineered recombinant JHMV encoding the Q600F or Q600V substitutions (rJ.S598Q600F and rJ.S598Q600V) (Fig. 3A) but could not develop a recombinant virus containing the Q600P substitution, most likely because the
substitution was detrimental to virus viability. As shown in Fig. 3B, both rJ.S598 Q600F and rJ.S598 Q600V exhibited similar in vitro growth kinetics as did JHMV encoding native S598 (rJ), indicating that the mutations do not greatly affect virus fitness. Initially, we showed that, similar to Q600Y (14), Q600F and Q600V enhanced the thermostability of the K b/peptide complexes \( t_m = 62.7 \pm 0.4^\circ C \) and \( t_m = 60.2 \pm 0.2^\circ C \), respectively) compared with wild-type K b/S598 (\( t_m = 56.2 \pm 1.9^\circ C \)) (Fig. 3C). Efficient K b/S598 complex formation required substitution of the cysteine of the peptide (RCQIFANI) with L-\( \alpha \)-aminobutyric acid (Aba, an isostereomer of cysteine). This modification at the P2-Cys does not affect epitope recognition (14). Thus Aba-modified peptides were used in both these analyses and the structural studies below.

We next examined the epitope-specific CD8 T cell responses in the brains of B6 mice intranasally infected with rJ or one of its variants. In mice infected with rJ.S598Q600Y (Fig. 4A, 4B),

### Table I. MRM transitions used to monitor for JHMV and VACV epitopes

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Sequence</th>
<th>Q1 m/z (Charge)</th>
<th>Q3 m/z (Ion)</th>
<th>Dwell Time (ms)</th>
<th>Optimal Collision Energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>B820–27 (VacV)</td>
<td>TSYKFEFSV</td>
<td>480.7 (+2)</td>
<td>609.3 (y5)</td>
<td>25</td>
<td><strong>28</strong></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>655.3 (SYKFE)</td>
<td>25</td>
<td><strong>30</strong></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>756.4 (b8)</td>
<td>25</td>
<td>22</td>
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<td></td>
<td></td>
<td></td>
<td>772.4 (y6)</td>
<td>25</td>
<td>23</td>
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<td></td>
<td></td>
<td></td>
<td>843.4 (b7)</td>
<td>25</td>
<td>23</td>
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<td></td>
<td></td>
<td></td>
<td>859.4 (y7)</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>S598 (JHMV)</td>
<td>RCQIFANI</td>
<td>482.8 (+2)</td>
<td>501.3 (b4)</td>
<td>40</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>648.3 (b3)</td>
<td>40</td>
<td>22</td>
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<td></td>
<td></td>
<td></td>
<td>719.4 (b8)</td>
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<td>22</td>
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<td></td>
<td></td>
<td></td>
<td>833.4 (b7)</td>
<td>40</td>
<td>22</td>
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<td></td>
<td></td>
<td></td>
<td>923.2 (y7)</td>
<td>40</td>
<td>22</td>
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<tr>
<td>S510 (JHMV)</td>
<td>CSLWNGPHL</td>
<td>513.7 (+2)</td>
<td>537.3 (y5)</td>
<td>40</td>
<td>20</td>
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<td></td>
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<td>723.4 (y4)</td>
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<td></td>
<td></td>
<td>836.4 (y7)</td>
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**FIGURE 2.** Increasing abundance of K b/S598 enhances functional avidity. B6 mice were i.p. infected with VacV-expressing epitopes S598 or S510 as a minigene or a soluble form of the S protein of JHMV or with rJ. (A–C) Magnitude and functional avidity of S598-specific CD8 T cell response in spleens of mice infected i.p. with VacV-S or VacV-S598 at day 7 p.i. (D) Functional avidity of S598-specific CD8 T cell response in spleens of mice infected i.p. with VacV-S or rJ. (E–G) Magnitude and functional avidity of S510-specific CD8 T cell response in spleens of mice infected i.p. with VacV-S or VacV-S510 at day 7 p.i. (H–J) Magnitude and functional avidity of B820–27-specific CD8 T cell response in spleens of mice infected i.p. with VacV-S, VacV-S598, or VacV-S510 at day 7 p.i. Data are representative of three independent experiments and presented as the means ± SEM (n = 3–4 mice/group). *p < 0.05, ***p < 0.001.
The growth kinetics of rJ, rJ.S Q600F, and rJ.S Q600V. Data are representative of substitution (rJ.S Q600F), or the Q600V substitution (rJ.S Q600V). (Fig. 4A–D and summarized in Fig. 4G). Additionally, cells primed by Q600F or S598 exhibited the greatest level of bidirectional cross-reactivity, with most of the S598-primed cells recognizing the Q600F variant (Fig. 4C and summarized in Fig. 4D). In contrast, only a small fraction of S598-primed CD8 T cells recognized Q600Y or Q600V peptide (Fig. 4A, 4E, upper right panels and summarized in Fig. 4B, 4F).

Because all of the S598 variants primed high-magnitude responses to S598, we next determined whether these responses exhibited higher functional avidity when reacted against S598 peptide than those induced by the native epitope. As shown in Fig. 5, all of the variants gave rise to CD8 T cell responses with lower EC50 than observed in rJ-infected mice. Cells primed by rJ.S598Q600Y or rJ.S598Q600F required ~10-fold less peptide (EC50 = 1.7 × 10^-8 and 1.3 × 10^-8 M, respectively) than did the rJ-primed cells (EC50 = 1.5 × 10^-7 M) to achieve a half-maximum response, whereas CD8 T cells selected by rJ.S598Q600V (EC50 = 2.2 × 10^-9 M) exhibited even greater potency for S598 (Fig. 5A, 5B). These results indicate that peptide Q600V is an ideal heteroclitic epitope, inducing a high-avidity, highly cross-reactive response to the native S598 epitope.

The results described in Fig. 2 suggest that higher Kb/S598 complex density resulted in a T cell response with higher functional avidity. To determine whether Kb/Q600V or Kb/Q600Y was also displayed at a higher density, we used two complementary approaches. First, we infected DC2.4 cells with VacV expressing S598, Q600V, or Q600Y as minigenes and assayed the expression of MHCI complexed with each peptide. Levels of peptide were lower from cells infected with VacV-S598 compared with cells infected with VacV expressing either variant (Fig. 6A), whereas levels of VacV-specific epitopes (B8 20–27) were similar in all infections. In a second approach, we reasoned that because S598 bound less stably to MHCI than did the variant peptides, it would exhibit lower surface levels after pulsing and washing. If this occurred, DCs coated with S598 would be expected to induce a lower CD8 T cell response after inoculation into mice, and this response should be of lower functional avidity. To address this possibility, we immunized mice with DCs pulsed with S598 or Q600V peptide and measured T cell responses. As shown in Fig. 6B, responses were detected to both peptides, but were much lower to S598 than Q600V; the response to S598 was so low that it precluded further analyses of functional avidity. Taken together, these results suggest that the surface levels of the epitopes with higher functional avidity (Q600Y and Q600V) were substantially higher than levels of S598 when expressed endogenously or after loading exogenously and a period of chase in vivo.

**FIGURE 3.** Development of rJ-expressing S598 variants exhibiting greater Kb binding. (A) Schematic depiction of recombinant JHMV expressing native S598 (rJ), the Q600Y substitution (rJ.SQ600Y), the Q600F substitution (rJ.SQ600F), or the Q600V substitution (rJ.SQ600V). (B) In vitro growth kinetics of rJ, rJ.SQ600F, and rJ.SQ600V. Data are representative of two independent experiments and presented as the means ± SEM. *p < 0.05, **p < 0.001 (rJ versus rJ.SQ600F); ***p < 0.001 (rJ versus rJ.SQ600V). (C) Summary of circular dichroism thermal denaturation scans of Kb/S598, Kb/Q600F, and Kb/Q600V complexes used to calculate Tm (parentheses). Data are from two independent experiments.

rJ.SQ600F (Fig. 4C, 4D), or rJ.SQ600V (Fig. 4E, 4F), a high frequency (35–40%) of brain-derived CD8 T cells recognized their cognate Ag with the variant S598-specific T cell response emerging as the immunodominant population. Concomitantly, the frequency of the S510-specific T cell response contracted relative to that induced in mice infected with wild-type rJ (summary data shown in Fig. 4B, 4D, 4F). Additionally, most of the CD8 T cells primed by each of the variants cross-reacted to native S598 (summarized in Fig. 4G). Approximately 75% of CD8 T cells primed by Q600Y or Q600F produced IFN-γ when stimulated with native S598 peptide (Fig. 4A–D and summarized in Fig. 4G). Most notably, Q600V induced the greatest proportion of cells with the desired specificity, with nearly all (~98%) of the Q600V-reactive T cells also recognizing native S598 (Fig. 4E, 4F, and summarized in Fig. 4G). Additionally, cells primed by Q600F or S598 exhibited the greatest level of bidirectional cross-reactivity, with most of the S598-primed cells recognizing the Q600F variant (Fig. 4C and summarized in Fig. 4D). In contrast, only a small fraction of S598-primed CD8 T cells recognized Q600Y or Q600V peptide (Fig. 4A, 4E, upper right panels and summarized in Fig. 4B, 4F).

Structures of H-2Kb in complex with the heteroclitic Q600V and Q600F peptides

To understand the structural requirements for the generation of heteroclitic peptides that elicit almost exclusively cross-reactive T cells, such as the Q600V analog, we determined the crystal structures of Kb/Q600V and Kb/Q600F and compared these to those of Kb/S598 and Kb/Q600Y (Fig. 7), which we previously determined (14). As in the thermostability experiments (Fig. 3C), the use of Aba-modified peptides was required for efficient Kb/S598 complex formation. The structures of the Kb molecule in complex with the Q600V and Q600F mutants of the S598 epitope were solved to a resolution of 2.0 and 2.3 Å, respectively (Table II). The structures of the pMHCI were consistent with the previously solved Kb structure in complex with the wild-type S598 epitope.
with a root mean square deviation (r.m.s.d.) of 0.25 Å of the Caα atoms on the α1α2 domains of the Kβ molecule (Fig. 7). The peptides adopted a similar conformation to that observed for the S598 epitope, namely P1-Arg, P4-Ile, and P7-Asn were solvent-exposed whereas P2-Aba, P3-Val/Phe, P5-Phe, and P8-Ile were buried in the Ag-binding cleft (Fig. 7). The peptides shared a highly similar structure, with an r.m.s.d. of 0.10 and 0.14 Å on the Caα atoms, respectively, for the Q600V (Fig. 7D) and Q600F (Fig. 7C) compared with the native S598 epitope. Thus, there were minimal structural perturbations in the pMHCI structures relative to the wild-type complex (Fig. 7E, 7F).

The three heteroclitic pMHC complexes exhibit a higher τm than does the native S598 pMHC complex (Fig. 3C and Ref. 14), which was associated with a higher number of intermolecular bonds observed in the pMHC structures, calculated using the CCP4 program suite as described in Materials and Methods (169, 175, 190, and 194 intermolecular bonds for S598, Q600V, Q600Y, and Q600F respectively, Table III). Next we addressed whether the higher number of bonds was a direct consequence of the amino acid substitution or involved other parts of the interface. For Q600Y and Q600V, the increased number reflected the amino acid substitution: P3-Y had almost twice as many bonds as P3-Q. This was also true for P3-V, but to a lesser extent. For Q600F, the situation was more complex, as we observed fewer bonds to P3-F compared with P3-Y, with a higher number of bonds spread over the epitope, reflecting modest side-chain movement. This was an indirect consequence of the P3-F mutation because all other parameters were the same (resolution, space groups, crystallization conditions).

The Kβ/Q600F and Kβ/Q600Y structures were very similar with an r.m.s.d. of 0.36 Å (Fig. 7E), with the large aromatic residues at P3 perfectly accommodated in the D pocket of the Kβ binding cleft (Fig. 8). In the Kβ/S598 complex, P3-Gln occupies a similar position (Fig. 8A); however, the larger side chains of tyrosine (Fig. 8B) and phenylalanine at P3 (Fig. 8C) contacted Arg155, an interaction that was not observed for the native S598 epitope or the Q600V epitope (Fig. 8). Notably, although the overall structures were similar, the substitution of the P3 residue by an aro-
Heteroclitic epitopes are a subset of peptide analogs known as altered-peptide ligands that were first used to enhance T cell responses to tumors (13, 47). Because tumor Ags are self-derived, antitumor T cells need to escape negative selection in the thymus and tend to have low affinity for the pMHC. In contrast, high-affinity pathogen-specific T cells are not eliminated by central tolerance mechanisms so that during an infection, T cells with a broad range of affinities for their cognate Ag are recruited into the response. Despite the presence of high-affinity pathogen-specific T cells in the periphery, some of these T cell responses exhibit low functional avidity. Under these circumstances, immunization with heteroclitic epitopes provides a useful approach to enhance the functional avidity of the pathogen-specific T cell response (14). T cells with increased functional avidity are associated with more efficient pathogen clearance in vivo (3, 8, 48) and, as we showed previously, with the prevention of CTL escape; for instance, the presence of the high–functional avidity Q600Y epitope was able to prevent CTL escape at epitope S510 in mice infected with rJ (14).

Our results now highlight a possible mechanism for how such heteroclitic peptides function: heteroclitic peptides enhance pMHC stability and consequent epitope dwell time on the cell surface, thereby increasing effective surface epitope density. This induces a T cell response with higher functional avidity. This suggests that increasing the Ag density presented to T cells that typically develop a low-avidity T cell response reprograms the T cell response and generates higher avidity responses. This is
consistent with antigenic competition during T cell expansion that can be overcome by increasing the Ag density (49).

In this study, the relationship between total pMHC being displayed and functional avidity was first demonstrated using mice infected with recombinant VacV expressing either the S protein or an S598 minigene. After infection, Kb/S598 complexes were not detected at quantifiable levels from cells infected with VacV-S (Fig. 1B, 2A), whereas high levels of Kb/S598 were present following VacV-S598 minigene infection. This increased expression of Kb/S598

Table II. Data collection and refinement statistics

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Values in parentheses are for the highest resolution shell.

Rmerge, a = ΣIobs − <Iall>/ΣIall

Rfree, a = Σobs||Fo|| − |Fcalc||Σobs||Fo|| for all data except ~5%, which were used for Rfree calculation.
complexes resulted in greatly enhanced functional avidity of the responding cells (Fig. 2B, 2C) and a modest increase in the magnitude of the S598-specific CD8 T cell response (Fig. 2A). These observations indicate that increased display of K\textsuperscript{b}/S598 provides sufficient signal to enhance the T cell response to this normally subdominant epitope and fosters the generation of high-avidity T cells. Increases in magnitude of response in vivo as epitope density increased have been reported previously (20, 50), but our results are in marked contrast with in vitro studies that showed diminished functional avidity of responding T cells as epitope density increased. The major difference between these reports and ours is that the T cell response to epitope S598 normally exhibits low functional avidity whereas epitopes with high–functional avidity responses were examined in the published studies. Consistent with this notion, enhancement in functional avidity of the response to epitope S510 was not observed when VacV-S510–infected mice were compared with those infected with VacV-S (Fig. 2E–G). This occurred even though analogously to K\textsuperscript{b}/S598, expression of the D\textsuperscript{b}/S510 complex was detectable by mass spectrometry in VacV-S510–infected cells but not when expressed from VacV-S (Fig. 1B, 1C).

To determine whether heteroclitic epitopes have the same physiological effect as increasing the pMHC abundance of the native epitope, we examined the capacity of two new and one established S598 analog to elicit high–functional avidity T cell responses that cross-reacted on the wild-type epitope (Figs. 4, 5). Viruses expressing each of the S598 analogs all elicited higher...
avidity T cell responses compared with the wild-type infection, with Q600V in particular generating T cells with almost two logs greater functional avidity when stimulated with the native peptide (Fig. 5). Moreover, the numbers of Kb/Q600Y or Kb/Q600V complexes were significantly greater than those of Kb/S598 from cells infected with VacV-expressing minigenes (Fig. 6A). Taken together, these results suggest that heteroclitic epitopes act in part by increasing the surface density of immunogenic and cross-reactive epitopes. Whether these results are valid for all or most heteroclitic epitopes and whether this is the sole or major basis of the heteroclitic effect of such epitopes will require further investigation. Thus, although we modified an anchor residue in the peptide that enhanced binding to the MHCI molecule, this modification may also have altered T cell binding (52), contributing to the heteroclitic effect that we observed.

The design of heteroclitic epitopes remains empiric, with the rules for determining optimal configuration not established. The two additional heteroclitic epitopes (Q600F and Q600V) that we identified augmented recognition of the native S598 epitope to a similar degree (Fig. 4C–F). As Tyr and Phe are structurally identified augmented recognition of the native S598 epitope to two additional heteroclitic epitopes (Q600F and Q600V) that we rules for determining optimal configuration not established. The variation may also have altered T cell binding (52), contributing to the heteroclitic effect that we observed.


References


