Modification of MHC Anchor Residues Generates Heteroclitic Peptides That Alter TCR Binding and T Cell Recognition


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Modification of MHC Anchor Residues Generates Heteroclitic Peptides That Alter TCR Binding and T Cell Recognition


Improving T cell Ags by altering MHC anchor residues is a common strategy used to enhance peptide vaccines, but there has been little assessment of how such modifications affect TCR binding and T cell recognition. In this study, we use surface plasmon resonance and peptide–MHC tetramer binding at the cell surface to demonstrate that changes in primary peptide anchor residues can substantially and unpredictably alter TCR binding. We also demonstrate that the ability of TCRs to differentiate between natural and anchor-modified heteroclitic peptides distinguishes T cells that exhibit a strong preference for either type of Ag. Furthermore, we show that anchor-modified heteroclitic peptides prime T cells with different TCRs compared with those primed with natural Ag. Thus, vaccination with heteroclitic peptides may elicit T cells that exhibit suboptimal recognition of the intended natural Ag and, consequently, impaired functional attributes in vivo. Heteroclitic peptide-based immune interventions therefore require careful evaluation to ensure efficacy in the clinic. The Journal of Immunology, 2010, 185: 2600–2610.
peptides can be less effective than natural peptides for the purposes of vaccination. Our results have important implications for the use of heteroclitic peptides and demonstrate the need for careful evaluation of this strategy on an individual basis before clinical application.

Materials and Methods

Generation of CD8+ T cell clones and primed mixed populations

CD8+ T cell clones were generated as described previously (5). The HLA A*0201-restricted Melan-A/MART-126–35 ELAGIGILTV-specific MEL5 clone and the HLA A*0201-restricted preproinsulin15–24 ALWGDPDAAAspecific 1E6 clone were described previously (22–24). PBMCs from an HLA A*0201+ donor were primed with either ELAGIGILTV or EAAGIGILTV as described previously (25).

Recognition screen and combinatorial peptide library scan

For the recognition screen, 2 × 10^5 1E6 CD8+ T cells were cultured in triplicate for 16 h with position 2 (p2) variants of the wild type preproinsulin15–24 peptide (ALWGDPDAAA) at a concentration of 1 μM. Supernatant was harvested and analyzed for TNF-α production by ELISA according to the manufacturer’s instructions (Life Technologies, Paisley, U.K.). The combinatorial peptide library (CPL) comprised a total of 9.36 × 10^4 ([10 + 19] × 19) different decamer peptides divided into 200 different peptide mixtures. For the CPL scan, 1E6 CD8+ T cells were washed and rested overnight in RPMI 1640 containing 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 2% heat-inactivated FCS (R2 medium; Life Technologies). In 96-well U-bottom plates, 6 × 10^4 1E6 CD8+ T cells were added and the assay was incubated overnight at 37°C. Subsequently, the supernatant was harvested and assayed for MIPβ by ELISA according to the manufacturer’s instructions (Abingdon, U.K.).

Generation of expression plasmids

The MEL5, MEL187.c5, and 1E6 TCRs were generated from the corresponding CD8+ T cell clones. All sequences were confirmed by automated DNA sequencing (Lark Technologies, Essex, U.K.). A disulfide-linked construct was used to produce the soluble domains (variable and constant) for both the TCR α and TCR β-chains (26, 27). The HLA A*0201 α-chain, tagged with a biotinylation sequence, and β2m were cloned and used to produce the pMHC I molecules. The MEL5, MEL187.c5, and 1E6 TCR α and TCR β-chains, the HLA A*0201 α-chain and β2m sequences were inserted into separate pGMT7 expression plasmids under the control of the T2 promoter (26).

Protein expression, refolding, and purification

Competent Rosetta DE3 Escherichia coli cells were used to produce the MEL5, MEL187.c5, and 1E6 TCR α and TCR β-chains, the HLA A*0201 α-chain, and β2m in the form of inclusion bodies using 0.5 mM IPTG to induce expression as described previously (26). For a 1 L TCF refold, 30 mg TCR α-chain was incubated at 37°C for 15 min with 10 mM DTT and added to cold refold buffer (50 mM TRIS pH 8.1, 2 mM EDTA, 2.5 M urea, 6 mM cysteamine hydrochloride, and 4 mM cystamine). After 15 min, 0.5 M β2m in the form of inclusion bodies was also incubated at 37°C for 15 min with 10 mM DTT. For a 1 L pMHC I refold, 30 mg HLA A*0201 α-chain was mixed with 30 μg β2m and 4 mg peptide at 37°C for 15 min with 10 mM DTT. The following peptides were used in separate refolds: ELAGIGILTV, EAAGIGILTV, ALWGDPDAAA, and ALWGDPDAAAspecific (heteroclitic changes are denoted in bold and underlined). This mixture was then added to cold refold buffer (50 mM TRIS pH 8.1, 2 mM cysteamine hydrochloride, and 4 mM cystamine). Refolds were mixed at 4°C for >1 h. Dialysis was performed against 10 mM TRIS pH 8.1, until the conductivity of the refolds was less than two millisiemens per centimeter. The refolds were then filtered, ready for purification steps. Refolded proteins were purified initially by ion exchange using a Poros30HQ column (GE Healthcare, Buckinghamshire, U.K.), then gel filtered into BIacore buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.05% (v/v) Surfactant P20) using a Superdex200HR column (GE Healthcare). Protein quality was analyzed by Coomassie-stained SDS-PAGE. Biotinylated pMHC I was prepared as described previously (28).

Surface plasmon resonance analysis

Binding analysis was performed using a BIACore T3000 (GE Healthcare) equipped with a CM5 sensor chip as reported previously (28). Between 200 and 400 response units (RU) of biotinylated pMHC I was immobilized on streptavidin, which was chemically linked to the chip surface; pMHC I was injected at a slow flow rate (10 μl/min) to ensure uniform distribution on the chip surface. Combined with the small amount of pMHC I bound to the chip surface, this reduced the likelihood of off-rate limiting mass transfer effects. The MEL5, MEL187.c5 and 1E6 TCRs were purified and concentrated to ~100 μM on the day of surface plasmon resonance (SPR) analysis to minimize TCR aggregation. For equilibrium analysis, eight serial dilutions were carefully prepared in triplicate for each sample and injected over the relevant sensor chip at a flow rate of 45 μl/min at 25°C. Results were analyzed using BIACalibration 3.1 (GE Healthcare), Microsoft Excel (Microsoft, Redmond, WA), and Origin 6.1 (OriginLab, Northampton, MA). The equilibrium binding constant (Kd) values were calculated using a nonlinear curve fit (y = [P1/x] + [P2 + x]).

pMHC I stability assays

For SPR-based pMHC I stability assays, ~1000 RU of biotinylated pMHC I was immobilized on streptavidin, which was chemically linked to the chip surface. The RU on each flow cell were monitored in real time over 4000 s at 25°C. Reductions in mass were analyzed using BIACalibration 3.1 to determine the stability of each pMHC I complex. For T2 cell-based pMHC I binding assays, the LCLxT-lymphoblastoid hybrid cell line 0.174×CEM. T2 was used. These cells, referred to as T2 cells (29), lack the transporter associated with Ag processing (TAP); thus, addition of exogenous binding peptide is required for stable expression of HLA A*0201 molecules on the cell surface. A total of 5×10^6 T2 cells per test were incubated in AIM V serum-free medium (Life Technologies) with various concentrations (0.01, 0.1, 1, 10, and 100 μM) of peptide at 26°C for 14–16 h, and then at 37°C for 2 h before staining for HLA A*0201 surface expression with the PE-labeled mAb BB7.2 (BD Biosciences, San Jose, CA). Duplicate samples for each condition were acquired using a BD FACSCantor flow cytometer (BD Biosciences). Data were analyzed with FlowJo software (TreeStar, Ashland, OR).

Cellular assays

The production of IFN-γ, TNF-α, and IL-2 by MEL5, MEL187.c5, and 1E6 CD8+ T cells was measured using a Cytometric Bead Array Th1/Th2 Assay Kit (BD Biosciences); degranulation was quantified using a flow cytometric assay to detect CD107a mobilization (30). In all experiments, 3×10^5 HLA A*0201-transfected C1R cells, pulsed with peptide at concentrations in the range of 10^{-7} to 10^{-15} M for 60 min at 37°C in 50 μl R2 medium, were mixed with 3×10^4 clonal CD8+ T cells in 50 μl R2 medium. The cells were incubated for 18 h at 37°C before analysis. Tetramer binding analysis was conducted using 5×10^6 clonal CD8+ T cells stained in minimal volume with 0.1 μg of the corresponding pMHC I component at 37°C for 30 min (31); cells were then washed with PBS and analyzed by flow cytometry. Tetramer association and dissociation assays were performed as described previously (32).

Flow cytometric sorting and molecular analysis of CD8+ T cell clonotypes

PBMCs primed in vitro with either ELAGIGILTV or EAAGIGILTV peptide were stained first at room temperature for 20 min with live-dead fixable Aqua (Life Technologies), and then at 37°C for 15 min with 1 μg of the corresponding HLA A*0201/ELAGIGILTV or HLA A*0201/EAAGIGILTV tetramer conjugated to allophycocyanin. Cells were then stained with anti-CD3-PECy7 and anti–CD8-Alexa Fluor 700 mAbs (BD Pharmingen, San Diego, CA) on ice for 20 min. Viable CD3+CD8+tetramer+ cells (median, 5000; range, 2781–5000) were sorted at 98% purity into 100 μl Ambion RNAlater (Applied Biosystems, Cheshire, U.K.) using a custom-modified BD FACSariaII flow cytometer (BD Biosciences Immunocytometry Systems). Molecular analysis of all expressed TRγ gene rearrangements was then performed as described previously (33). The ImMunoGeneTics nomenclature is used throughout this report (34).

Results

Mutation at a primary MHC I anchor residue can substantially alter T cell function

In this study, we examined the effect of MHC anchor residue changes on T cell recognition (refer to Table 1 for current HLA A*0201-restricted natural human cancer Ags and their heteroclitic variants). We
reasoned that the examination of such outcomes would be best explored initially in the context of a low-affinity TCR–pMHC I interaction, which might facilitate the measurement of subtle effects. Autoimmune disease provides such a context, and we therefore focused on the IE6 CD8+ T cell clone, which is specific for the HLA A*0201-restricted human preproinsulin15–24 peptide ALWGDPDAAA. This clone was recently derived from the peripheral blood of a patient with T1D; a clone with an identical TCR was subsequently isolated from the same patient 1 y later, thereby demonstrating the long-term persistence in vivo of T cells with this specificity (22). In a recognition screen using peptides with all possible proteogenic amino acids at p2, which is a primary anchor residue for peptides that bind HLA A*0201, glutamine (Q) was found to be the optimal residue for the induction of IE6 T cell activation (Fig. 1A). This result was unexpected because Q is known to be a suboptimal anchor for HLA A*0201 at this position (14, 15). We confirmed that Q was the preferred residue for IE6 T cell recognition at this position using a p2 CPL scan (Fig. 1B). Furthermore, similar effects were observed in assays with different functional readouts. Thus, the AQWGPDPAAAP2 variant induced greater secretion of MIP1β, IFN-γ, TNF-α, and IL-2 (Fig. 2A–D) and enhanced T cell degranulation (Fig. 2E) at low peptide concentrations. The difference in activation was most marked with IL-2 production (5); AQWGPDPAAA induced IL-2 production from IE6 T cells at concentrations of exogenously applied peptide that were 100-fold lower compared with the 2L wild type peptide (Fig. 2D). This unexpected finding, that substitution with a suboptimal anchor residue at a primary MHC I binding site generated a substantially improved T cell agonist peptide, warranted further investigation.

**Mutation at a primary MHC I anchor residue can substantially alter TCR binding**

Analysis of pHLA A*0201 stability on the surface of T2 cells showed that the AQWGPDPAAA (2Q) variant exhibited suboptimal binding compared with ALWGDPDAAA (Fig. 3, inset). To examine this effect in more detail, we developed a sensitive SPR-based assay to examine pMHC I stability in real time. Biotinylated HLA A*0201/ALWGDPDAAA and HLA A*0201/AQWGPDPAAA were immobilized on the surface of separate BIAcore CM5 chips using streptavidin-biotin coupling. A reduction in RU values over time was observed, corresponding to a reduction in mass at the chip surface (Fig. 3). The loss of peptide from pMHC I complexes cannot wholly account for this RU reduction, because of the relatively small mass of the peptide (~1 kDa). Instead, we reasoned that the majority of this mass reduction was due to the dissociation of β2m from the MHC I H chain caused by destabilization of the pMHC I complex upon loss of the antigenic peptide; thus, this decay process represents the t1/2 of peptide binding. Consistent with this hypothesis, the ALWGDPDAAA peptide, which contains a preferred HLA A*0201 binding residue at p2, displayed a slower dissociation rate compared with the AQWGP-DPAAA peptide, which incorporates a suboptimal p2 anchor residue (Fig. 3). This new assay showed that the t1/2 of the wild type HLA A*0201/ALWGDPDAAA complex was 4.8-fold longer than the corresponding HLA A*0201/AQWGPDPAAA complex (Fig. 3). Thus, the 2Q peptide elicited substantially better activation of IE6 T cells despite reduced MHC I binding.

We next examined TCR–pMHC I affinity to investigate the molecular basis for the enhanced agonist properties of the AQWGPDPAAA peptide. Soluble IE6 TCR bound to HLA A*0201/AQWGPDPAAA with a substantially greater affinity (KD ≈ 8.4 × 10⁻⁴ M) compared with HLA A*0201/ALWGDPDAAA (KD ≈ 2.7 × 10⁻⁴ M) in SPR experiments (Fig. 4A, 4B). These

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**Table I. List of current natural human cancer Ags and their related heteroclitic peptides**

<table>
<thead>
<tr>
<th>Tumor-Associated Protein Ag</th>
<th>HLA Allele</th>
<th>Natural Peptide</th>
<th>Heteroclitic Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melan-A26-35</td>
<td>HLA A*0201</td>
<td>EAAIGILTV</td>
<td>ELAIGILTV(9)</td>
</tr>
<tr>
<td>NY-ESO-1157-165</td>
<td>HLA A*0201</td>
<td>SLLMWTVQ</td>
<td>SLLMWTVQ(3)</td>
</tr>
<tr>
<td>gp100280-217</td>
<td>HLA A*0201</td>
<td>ITDQVPSV</td>
<td>ITDQVPSV(6)</td>
</tr>
<tr>
<td>gp100340-288</td>
<td>HLA A*0201</td>
<td>YLEPGFV</td>
<td>YLEPGFV(6)</td>
</tr>
<tr>
<td>gp100340-342</td>
<td>HLA A*0201</td>
<td>KXQVQYWQV</td>
<td>KXQVQYWQV(6)</td>
</tr>
<tr>
<td>HER-1/neu169-377</td>
<td>HLA A*0201</td>
<td>KIPGSLAFY</td>
<td>KIPGSLAFY(4)</td>
</tr>
<tr>
<td>PSA178-187</td>
<td>HLA A*0201</td>
<td>VISNDVCAQV</td>
<td>VISNDVCAQV(8)</td>
</tr>
</tbody>
</table>

Anchor residue modifications are in boldface and underlined.

---

**FIGURE 1.** A, Recognition of all p2 variants in the preproinsulin15–24 peptide sequence by the IE6 CD8+ T cell clone; 2 × 10⁴ IE6 T cells were cultured in triplicate for 16 h with p2 variants of the preproinsulin15–24 peptide at a concentration of 1 μg/ml. Supernatants were then harvested and assayed for TNF-α production by ELISA. Results are representative of three independent assays. B, A p2 CPL screen. Each 10-mer peptide pool was defined by a fixed amino acid at p2 and a random equimolar mixture of all 20 proteogenic L-amino acids, except cysteine, at the remaining nine positions (OₓXₙ). Cysteine was included at the fixed position (O), but omitted from the degenerate positions (X) to limit the problem of oxidation. In each 18-h assay, 3 × 10⁴ IE6 CD8+ T cells were exposed in the presence of individual peptide mixtures to 6 × 10⁴ CIR target cells stably transfected with HLA A*0201. Supernatants were then harvested and assayed for MIP1β production by ELISA. In each case, the star indicates the wild type p2 leucine residue.
A
tetramer stained 1E6 T cells with greater intensity than the HLA
upon loss of the antigenic peptide. HLA A
over 4000 s at 25˚C. The reduction in RUs over time reflects dissociation
on to each flow cell via a biotin-streptavidin linker. The assay was monitored
A
peptides. In all experiments, 3
pulsed with peptide at concentrations in the range of 10
m
h) dissociated more slowly than HLA A
Real-time pMHC I stability assay comparing the binding of
FIGURE 3.
A
with an affinity more than twice that of the corresponding HLA
Heteroclitic complex (Fig. 8
MEL187.c5 T cell clone exhibited substantially better staining
with greater stability compared with EAAGIGILTV , has been
studied tumor-associated epitope to date (9, 35–37). The E
anchor residue can substantially alter TCR binding.

Stability and specificity investigations in the clinically relevant
Melan-A/MART-126–35 system
To extend our findings to a clinically relevant system, we exam-
cined CD8+ T cell responses to the HLA A
resticted Melan-
A/MART-126–35 peptide EAAGIGILTV , which is the most widely
studied tumor-associated epitope to date (9, 35–37). The ELAGI-
GILTV (2L) heteroclitic variant of the Melan-A/MART-126–35 peptide,
which contains the preferred HLA A
motif and binds with greater stability compared with EAAGIGILTV , has been
widely adopted in this system because it induces far greater CD8+
T cell expansions than the natural peptide (21). The 2L heteroclitic
peptide is also used exclusively in Melan-A/MART-126–35 pMHC I
multimers because of their greater stability. We confirmed the
enhanced stability of HLA A
/ELAGIGILTV in a T2 binding
assay (Fig. 6, inset). This result was further confirmed using our
novel SPR pMHC I stability assay, which showed that the t/2 of
HLA A
/ELAGIGILTV was 5.3-fold longer than the corre-
MEL187.c5 T cell clone responded more strongly to HLA A
with TCR–pMHC I binding, although such buried
assays. As predicted, the two TCRs exhibited differential binding to the HLA
E
A
B
A
E
C
D
FIGURE 2. Activation of the 1E6 CD8+ T cell clone in response to wild
type ALWGPDPAAAl (ALW) and heteroclitic AQWGPDPAAAl (AQW)
peptides. In all experiments, 3 \times 10^6 HLA A
-transfected C1R cells, pulsed with peptide at concentrations in the range of 10^{-3} to 10^{-11} M for 60 min at 37˚C in 50 \mu R2 medium, were mixed with 3 \times 10^6 1E6 CD8+ T cells in 50 \mu R2 medium. The cells were incubated for 18 h at 37˚C before
analysis. A, MIPβ ELISA assay. Error bars represent one SD and, in most
cases, are smaller than the plot symbols. B–D, Cytometric bar bead showing IFN-γ, TNF-α, and IL-2 production. E, Degranulation (CD107a) assay.
data clearly show that anchor residue modification can have a profound effect on TCR–pMHC I binding, although such buried
molecules do not usually contact the TCR during Ag recognition.
In addition, we examined ligand binding at the T cell surface using
pMHC I multimers because of their greater stability. We confirmed the
enhanced stability of HLA A
/ELAGIGILTV in a T2 binding
assay (Fig. 6, inset). This result was further confirmed using our
novel SPR pMHC I stability assay, which showed that the t/2 of
HLA A
/ELAGIGILTV was 5.3-fold longer than the corre-
MEL187.c5 T cell clones, MEL5 and MEL187.c5, using five distinct
functional readouts (Fig. 7). The MEL187.c5 clone responded
more strongly to HLA A
with TCR–pMHC I binding, although such buried
assays. As predicted, the two TCRs exhibited differential binding to the HLA
E
A
B
A
E
C
D
FIGURE 3. Real-time pMHC I stability assay comparing the binding of
ALWGPDPAAAl (ALW) and AQWGPDPAAAl (AQW) peptides to HLA
A
using SPR. Approximately 1000 RUs of each pMHC I was attached
to each flow cell via a biotin-streptavidin linker. The assay was monitored
over 4000 s at 25˚C. The reduction in RUs over time reflects dissociation
of the antigenic peptide. HLA A
/ALWGPDPAAAl (t1/2 ~ 87 h) dissociated more slowly than HLA A
/AQWGPDPAAAl (t1/2 ~ 18 h). Inset, Cellular pMHC I stability assay. Live T2 cells were pulsed with 10 \mu M peptide and pHLA A
was quantified on the cell surface by flow
cytometry using a specific PE-labeled mAb. Exogenous application of
ALWGPDPAAAl (ALW) exhibited superior pMHC I surface stabilization compared with AQWGPDPAAAl (AQW). These data show that the wild
type preproinsulin15–24 peptide with leucine at p2 forms a more stable
pHLA-A
complex than does the corresponding 2Q peptide.
the validity of using Melan-A/MART-126–35 heteroclitic peptides in vaccine trials (35, 37–39). Specifically, this binding disparity suggests that the heteroclitic ELAAGIGILTV peptide might prime T cells with different constituent clonotypes that exhibit suboptimal recognition of the natural Ag presented on the tumor cell surface. To examine this possibility, we dissected the clonotypic composition of CD8+ T cell populations specific for HLA A*0201/EAAGIGILTV and HLA A*0201/ELAAGIGILTV after in vitro priming of PBMCs with the respective peptides. In all cases, Ag-specific CD8+ T cells primed with either EAAGIGILTV or ELAAGIGILTV were identified using the corresponding pHLA A*0201 tetramers and sorted by flow cytometry to >98% purity. A quantitative molecular analysis of all expressed TRB gene products was then conducted using a template-switch anchored RT-PCR (33). To minimize the effect of stochastic processes during in vitro priming, we pooled all TRB gene transcripts specific for either HLA A*0201/EAAGIGILTV (260 sequences) or HLA A*0201/ELAAGIGILTV (141 sequences) derived from one HLA A*0201 donor at different time points and weighted each distinct sequence equally regardless of frequency (Figs. 10, 11). A common bias toward TRBV4-1, TRBV6-2/6-3, TRBV6-5, TRBV27, and TRBV28 was observed in the CD8+ T cell repertoires primed by both HLA A*0201/EAAGIGILTV and HLA A*0201/ELAAGIGILTV. In contrast, TRBJ gene usage was more diverse in both repertoires, although HLA A*0201/EAAGIGILTV-specific clonotypes exhibited preferential usage of TRBJ1-5. Glycine residues were well represented within the CDR3 sequences, but no prevalent motifs were apparent. However, HLA A*0201/EAAGIGILTV-specific clonotypes tended to use shorter CDR3s compared with HLA A*0201/ELAAGIGILTV-specific clonotypes (Figs. 10, 11). Notably, only 15 clonotypes were shared between the two concatenated datasets; in contrast, there were 62 distinct clonotypes within the HLA A*0201/EAAGIGILTV-primed repertoire and 39 distinct clonotypes within the HLA A*0201/ELAAGIGILTV-primed repertoire. These data indicate that differences in T cell fine specificity affect clonotype selection, at least in vitro, and are consistent with the biophysical and functional data derived from different CD8+ T cell clones specific for Melan-A/MART-126–35 (Figs. 7–9).

Discussion

There are currently a number of anchor residue-modified peptides undergoing clinical assessment in cancer vaccine trials; these heteroclitic peptides target Melan-A/MART-1 (9), NY-ESO-1 (3), gp100 (6), HER-1/neu (4) and PSA (8). The purpose of peptide modification at anchor residues is to enhance the stability of MHC binding and thereby increase immunogenicity, with the inherent assumption that responding T cells will maintain specificity for the wild type Ag. Although there is evidence that such heteroclitic peptides can induce stronger T cell responses compared with their wild type counterparts, the induced T cell expansions do not appear to mount robust tumor-specific responses (40–

**FIGURE 4.** SPR equilibrium binding of soluble 1E6 TCR to (A) HLA A*0201/ALWGPDPAAA (A2-ALW) and (B) HLA A*0201/AQWGPDPAAAA (A2-AQW). The mean response for each concentration is plotted (n = 2). The equilibrium $K_D$ values were calculated assuming 1:1 Langmuir binding and were plotted using a nonlinear curve fit ($y = [P_1 x]/[P_2 + x]$). These data show that TCR binding affinity is directly related to the sensitivity of a T cell for cognate Ag. The CDR3 sequences do not appear to mount robust tumor-specific responses.

**FIGURE 5.** Wild type and heteroclitic pMHC I tetramer staining of 1E6, MEL5, and MEL187.c5 CD8+ T cell clones. A, HLA A*0201/ALWGPDPAAA (ALW) and HLA A*0201/AQWGPDPAAAA (AQW) tetramer staining of the 1E6 CD8+ T cell clone. B, HLA A*0201/EAAGIGILTV (EAA) and HLA A*0201/ELAAGIGILTV (ELA) tetramer staining of the MEL5 CD8+ T cell clone. C, HLA A*0201/EAAGIGILTV (EAA) and HLA A*0201/ELAAGIGILTV (ELA) tetramer staining of the MEL187.c5 CD8+ T cell clone. The MFI of tetramer staining is indicated in each case. These data show that tetramer staining avidity is directly related to monomeric TCR–pMHC I affinity. MFI, mean fluorescence intensity.
Commencing our study with the HLA-A*0201/preproinsulin$_{15-24}$ epitope, one of very few defined TCR–pMHC I systems in autoimmune disease, we discovered that a cognate CD8$^+$ T cell clone from a patient with T1D activated more readily in response to an anchor residue-modified peptide containing a L2Q substitution (Figs. 1, 2). This result was unexpected, given that Q is not an accepted residue at a primary HLA A*0201 binding residue at p2 (consensus sequence: $x_{1-6}LxxxxxV/L$) (14, 15). Indeed, using standard techniques, we confirmed that AQWGPDPAAA bound to HLA A*0201 with reduced affinity compared with the natural ALWGPDPAAA peptide (Fig. 3).

Furthermore, we developed a sensitive SPR-based assay to examine pMHC I complex stability in real time. Using this approach, we found that the L2Q substitution reduced the pHLA A*0201 complex $t_{1/2}$ from $\sim$87 to $\sim$18 h. The finding that a cognate CD8$^+$ T cell clone preferred the modified HLA A*0201/AQWGPDPAAA Ag, despite the substantial reduction in peptide binding and complex stability, suggested that the clonotypic TCR might bind the heteroclitic form with greater affinity compared with the wild type HLA A*0201/ALWGPDPAAA molecule. Indeed, soluble IE6 TCR bound HLA A*0201/AQWGPDPAAA with a substantially greater affinity ($K_D \approx 8.4 \times 10^{-5} \text{ M}$) compared with HLA A*0201/ALWGPDPAAA ($K_D \approx 2.7 \times 10^{-4} \text{ M}$) in SPR equilibrium binding experiments (Fig. 4A, 4B). This difference in TCR binding was also evident at the cell surface in experiments with pMHC I tetramers (Figs. 4C, 4D, 5A). Thus, we concluded that amino acid substitution at a primary HLA A*0201 anchor residue (i.e., p2) (43, 44) could enhance T cell recognition and TCR binding despite causing a substantial reduction in peptide affinity for MHC I. This striking observation led us to consider that the converse scenario might also occur, a proposition that could have important implications for the use of heteroclitic peptide-based vaccines in cancer immunotherapy.

The ELAGIGILTV heteroclitic variant of Melan-A/MART-1$_{126-35}$ is known to bind HLA A*0201 with higher affinity than the wild type EAAGIGILTV peptide (Fig. 6) (45). This enhanced stability is thought to explain why HLA A*0201/ELAGIGILTV is a more potent T cell agonist (21, 45), because Ag density on the cell surface and the duration of stimulus will be substantially increased. Two distinct CD8$^+$ T cell clones specific for Melan-A/MART-1$_{126-35}$, MEL5 and MEL187.c5, were able to distinguish between the EAAGIGILTV and ELAGIGILTV peptides in a wide range of functional assays (Fig. 7). Biophysical analysis showed that the MELS TCR bound HLA A*0201/EAAGIGILTV with greater affinity (>2.5-fold) than HLA A*0201/ELAGIGILTV (Fig. 8A, 8B).
This binding preference was also evident at the cell surface (Figs. 5B, 8C, 8D). Conversely, the MEL187.c5 TCR bound HLA A*0201/EAAGIGILTV with lower affinity (2-fold) than HLA A*0201/ELAGIGILTV (Fig. 9B). Again, this difference was apparent at the cell surface (Figs. 5C, 9C, 9D). Thus, distinct TCRs can recognize an anchor residue-modified heteroclitic peptide differently in the Melan-A/MART-126–35 system.

To date, all our experiments using altered peptide ligands or mutated TCRs with defined biophysical binding properties (5, 46) (unpublished) have shown that the most functionally sensitive T cells express TCRs with the highest affinity for cognate Ag. Furthermore, we have shown that TCRs raised against tumor-specific peptides bind cognate pMHC Ag with affinities that are substantially weaker than those raised against pathogen-derived peptides (18). In the current study, it is noteworthy that MEL187.c5 TCR binding to both forms of the Melan-A/MART-126–35 Ag and MEL5 TCR binding to HLA A*0201/ELAGIGILTV occur within or just below the range reported for other MHC I-restricted tumor-specific TCRs (KD ≈ 1.1 × 10⁻⁸ M to 3.4 × 10⁻⁶ M) (18). In contrast, the MEL5 TCR bound HLA A*0201/EAAGIGILTV with a substantially higher affinity (KD ≈ 6.4 × 10⁻⁶ M), which falls within the range typically observed for MHC I-restricted pathogen-specific TCRs (18). Therefore, it is likely that CD8⁺ T cells with identical or similar specificity to MEL5 represent more desirable targets for optimal adoptive therapy and vaccination trials compared with MEL187.c5-like T cells.

Structural evidence suggests that substitution of alanine to leucine at the p2 anchor residue does not substantially alter Melan-A/MART-126–35 peptide conformation in the uncomplexed pHLA A*0201 molecule (47, 48). However, a number of recent studies have shown that peptide conformation can be substantially altered during TCR docking (49–51). We have recently solved the crystal structure of the MEL5 TCR in complex with HLA A*0201/ELAGIGILTV (18). When compared with the structure of the MEL5 TCR in complex with HLA A*0201/EAAGIGILTV (Fig. 5A, 5B), the TCR is induced to a more native-like conformation that is typical of MHC I-restricted pathogen-specific TCRs (18). Therefore, it is likely that CD8⁺ T cells with identical or similar specificity to MEL5 represent more desirable targets for optimal adoptive therapy and vaccination trials compared with MEL187.c5-like T cells.
This structure showed that the MEL5 TCR α-chain makes a number of electrostatic interactions with the N-terminus of the peptide, particularly residues 1E, 2L, and 4G. The electrostatic interaction between the MEL5 TCR and the main chain of 2L is of particular note, because it shows that TCR-anchor residue contacts can contribute to Ag specificity. It is likely that peptides with suboptimal anchor residues may be more flexible within the MHC binding groove, thereby enabling them to

FIGURE 10. Concatenated molecular analysis of CD8+ T cell clonotypes specific for the Melan-A/MART-126–35 Ag. PBMCs from an HLA A*0201+ donor were primed at two different time points with (A) EAAGIGILTV and (B) ELAGIGILTV peptides. Each distinct sequence for each specificity was counted once, irrespective of clonal frequency, to minimize bias arising from preferential clonotypic expansions in vitro. Panels show TRBV and TRBJ gene usage, and CDR3 aa sequence. Clonotypes that are common to both repertoires are color-coded.

ELAGIGILTV (24). This structure showed that the MELS TCR α-chain makes a number of electrostatic interactions with the N-terminus of the peptide, particularly residues 1E, 2L, and 4G. The electrostatic interaction between the MELS TCR and the main chain of 2L is of particular note, because it shows that TCR-anchor residue contacts can contribute to Ag specificity. It is likely that peptides with suboptimal anchor residues may be more flexible within the MHC binding groove, thereby enabling them to
form subtly different conformational motifs that could have an effect on the fine specificity of different T cell clones. In the case of the MEL5 TCR, increased flexibility in this region of the peptide may allow for stronger, or even new, TCR–peptide contacts that could explain the enhanced TCR affinity and preferential Ag sensitivity of MEL5 for HLA A*0201/EAAGIGILTV compared with HLA A*0201/E\textsubscript{L}AGIGILTV (Figs. 7, 8).

Current wisdom has resulted in the use of heteroclitic peptides for the majority of HLA A*0201-restricted tumor-associated epitopes (Table I). The differential binding of TCRs to such peptides, as demonstrated in this study, gives cause for concern because it may result in the priming of T cell populations that recognize the natural Ag suboptimally. Indeed, we observed that different TCR repertoires were primed in vitro by the EAAGIGILTV and EL\textsubscript{L}AGIGILTV peptides, even from the same PBMC sample under otherwise identical conditions (Figs. 10, 11). These differences likely reflect clonotypic preferences for either form of the Melan-A/MART-1\textsubscript{26–35} Ag. Our observations in this system are timely given recent elegant in vivo studies showing that unmodified self-Ag triggers human CD8\textsuperscript{+} T cells with stronger tumor reactivity than the altered EL\textsubscript{L}AGIGILTV Ag (21). These studies compared the CD8\textsuperscript{+} T cell responses and clonotypic composition of antigenic peptides to improve HLA A*0201 binding can alter TCR fine specificity with attendant functional consequences at the cellular level. The heteroclitic EL\textsubscript{L}AGIGILTV peptide has been used in a number of clinical trials (37, 39), but these trials have been abandoned because of a low likelihood of success (58, 59). Our observation that TCRs can distinguish between the heteroclitic EL\textsubscript{L}AGIGILTV peptide used in vaccination and the wild type EAAGIGILTV sequence found on the surface of melanoma cells provides a biophysical reason for this failure. Indeed, our data synergize well with recent studies in which patients with melanoma were vaccinated using either the EAAGIGILTV or EL\textsubscript{L}AGIGILTV peptides (21). These seminal studies revealed that, although analog vaccination was immunologically weaker than heteroclitic vaccination,
the responding T cell repertoires were therapeutically superior in terms of avidity and effector function (21). We therefore conclude that the use of anchor residue-modified, heterocyclic peptides requires careful re-evaluation to ensure that primed T cells exhibit optimal specificity and sensitivity for the intended target.

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