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Peptide length determines the outcome of TCR/peptide-MHCI engagement

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**Key Points**

- MHCI-restricted TCRs exhibit an explicit preference for a single MHCI-peptide length.
- Effective CD8+ T-cell immunity can only be achieved by length-matched Ag-specific T-cell clonotypes.

Introduction

MHC class I (MHCI) molecules present short peptide fragments at the cell surface, enabling inspection of the intracellular proteome by CD8+ T cells. This elegant display system allows clearance of cells that exhibit abnormal biochemistry or express non-self, pathogen-derived proteins. The MHCI complex contains an Ag-binding cleft specifically designed to cradle a single peptide fragment. CD8+ T cells engage pMHCI molecules via their clonotypic αβ-TCRs, which are encoded by variable (V), diversity (D), joining (J), and constant (C) gene fragments. These fragments recombine, with additional N-nucleotide insertions/deletions at V(D)J junctions, to produce a theoretical repertoire of 10^15-10^20 unique receptors.1-3 As a consequence of size limitations and thymic selection, the in vivo TCR repertoire has been estimated at 2.5 × 10^7 receptors.4,5 The focal point of TCR diversity is manifested in the Ag-binding site, which comprises 6 highly flexible complementarity determining regions (CDRs).5-7

TCRs are inherently cross-reactive toward pMHC molecules. Indeed, a recent comprehensive analysis of TCR cross-reactivity demonstrated that a single TCR is capable of recognizing more than one million different peptides of defined length presented in the context of a single MHCI restriction element.7 This feature explains how the naive TCR repertoire, which is dwarfed by the potential number of pMHC molecules that could be encountered in nature, can successfully provide broad antigenic coverage. TCR cross-reactivity is essential for normal processes such as positive selection in the thymus and survival of naive T cells in the periphery. A consequence of this cross-reactivity, however, is a high frequency of T cells capable of recognizing distinct peptides in the context of non-self MHC, a phenomenon known as alloreactivity.8-10 In addition, several studies have demonstrated the induction of autoimmune with exogenous microbial peptides9 and, more recently, the ability of a single TCR to recognize both pMHC and pMHCI Ags.10-12 The huge scope for CDR loop flexibility on binding to pMHCI13,14 and the ability of the TCR to force changes in the shape of the peptide15 provide a structural explanation for the ability of a single TCR to recognize a multitude of different pMHC shapes.16-18

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Initially, it was thought that MHCI presentation was restricted to peptides 8-10 amino acids in length, but it is now established that peptides > 10 amino acids in length play a significant role in immune surveillance by CD8+ T cells. As such, the TCR repertoire must be able to recognize all possible MHCI-associated peptide lengths (8-14 amino acids) to achieve effective immunity. Given the highly cross-reactive nature of TCR recognition, as well as the inherent bias that TCRs are considered to have for the MHC, it seemed reasonable to assume that TCRs can adapt to and recognize peptides of a different length bound to the same allotype. To address this assumption, we synthesized 6 combinatorial peptide libraries (CPLs) ranging from 8-13 amino acid residues in length and measured TCR recognition across an array of human MHCI-restricted CD8+ T-cell clones. These αβ-T-cell clones were raised against a range of pMHCI Ags derived from both foreign and self-Ags. Unexpectedly, we found that strict limits exist to TCR plasticity in that each receptor is predisposed to engage peptides of a defined length. Thus, while any given TCR is capable of recognizing tens of thousands, if not millions, of different peptides, these peptides must be of an explicit length that varies depending on the individual TCR.

**Methods**

**Cells**

The following HLA A*0201–restricted CD8+ T-cell clones were used in this study: 1E6, specific for the preproinsulin-derived epitope ALWGPDPAAA (residues 15-24); ILA1, specific for the human telomerase reverse transcriptase (hTERT)-derived epitope ILAKFLHWL (residues 540-548); and MEL5, specific for the Melan-A– derived epitope ELAGIGILTV (residues 26-35). In addition, the following HLA B*3508–restricted CD8+ T-cell clones were used: SB14, specific for the EBV-derived EBNA-1 epitope HPVGEADYFEY (residues 407-417); SB10, specific for the HCMV-derived pp65 epitope CPSQEPMSIYYV (residues 103-114); and SB27, specific for the EBV-derived BZLF1 epitope LPEPLPQGQLTAY (residues 52-64). The recognition parameters of these CD8+ T-cell clones are detailed in Table 1. C1R-HLA A*0201 target cells and T2-HLA B*3508 target cells were generated as described previously.

### Sizing scan

The following mixtures were used to define the MHCI-peptide length preference of individual TCRs: X8, X9, X10, X11, and X12 (where X is any of the 19 proteogenic L-amino acids excluding cysteine; Pepscan Presto). Sizing scan parameters are detailed in Table 2. CD8+ T-cell clones were washed and rested overnight in RPMI 1640 medium containing 100 U/mL of penicillin, 100 μg/mL of streptomycin, 2mM l-glutamine, and 2% heat-inactivated FCS (all Life Technologies). In 96-well, U-bottom plates, 6 × 10^4 target cells were incubated with sizing scan mixtures (at 1mM) in duplicate for 2 hours at 37°C. After peptide pulsing, 3 × 10^4 clonal CD8+ T cells were added and the assay was incubated overnight at 37°C. The supernatant was assayed by MIP1β ELISA (R&D Systems).

### Peptide titration assays: MIP1β ELISA and quantification of T-cell cross-reactivity

Six × 10^4 target cells were pulsed with peptide at the indicated concentrations for 2 hours at 37°C. Three × 10^4 clonal CD8+ T cells were added and the assay was incubated overnight at 37°C. Subsequently, the supernatant was assayed for MIP1β by ELISA (R&D Systems). Functional sensitivity is expressed by the pEC50 for each peptide with respect to the TCR. This is defined as minus 1 times the base-10 logarithm (p) of the 50% efficacy concentration (EC50); a greater functional sensitivity is indicated by a larger pEC50 value, which was estimated as described previously. T-cell cross-reactivity was quantified by CPL-based importance sampling as described previously.

### pMHCI staining and flow cytometry

Soluble biotinylated pMHCI monomers were produced as described previously. Tetrameric pMHCI reagents (tetramers) were constructed by the addition of PE-conjugated streptavidin (BD Biosciences). Duplicate samples for each condition were acquired using a FACSCanto II flow cytometer (BD Biosciences). Data were analyzed with FlowJo Version 9.5.3 software (TreeStar).

### Combinatorial peptide library scans

The 8mer, 9mer, 10mer, 11mer, 12mer, and 13mer CPLs were synthesized in positional scanning format (Pepscan). CPL parameters are detailed in Table 3. For CPL screening, CD8+ T-cell clones were washed and rested overnight in RPMI 1640 medium containing 100 U/mL of penicillin, 100 μg/mL of streptomycin, 2mM l-glutamine, and 2% heat-inactivated FCS (Life Technologies). In 96-well, U-bottom plates, 6 × 10^4 target cells were incubated with various library mixtures (at 100μg/mL) in duplicate for 2 hours at 37°C. After peptide pulsing, 3 × 10^4 clonal CD8+ T cells were added and the assay was incubated overnight at 37°C. The supernatant was assayed by MIP1β ELISA (R&D Systems).

### Crystallization, structure determination, and refinement

HLA A*0201 in complex with ILA-KFLHWL (A2-ILA) was refolded as described previously and then concentrated to 10 mg/mL in 10mM MES and 10mM NaCl. Screens were set up in 96-well Intelliplates (Art Robbins)
Instruments) by the crystal Phoenix robot (Art Robbins Instruments) applying the sitting drop vapor diffusion technique. Next, 0.2 μL of HLA A*0201 ILAKLFHRL and 0.2 μL of crystallization buffer were dispensed into the small reaction well and 60 μL of crystallization buffer was dispensed into the large reservoir. Intelli-plates were then sealed, incubated at room temperature in a crystallization incubator (Molecular Dimensions), and analyzed for crystal formation. Crystals selected for further analysis grew in 0.1M MES (pH 7), 15% PEG 4000, 0.2M (NH4)2SO4, and were cryoprotected with ethylene-glycol to 25% and then flash cooled in liquid nitrogen in Litho loops (Molecular Dimensions). Diffraction data were collected on beamline 103.1 using a fixed wavelength of 0.9163 Å at the Diamond light source, Oxford, with a Pilatus 2M detector. Using a rotation method, 400 frames were recorded, each covering 0.5° of rotation. Reflection intensities were estimated with the MOSFLM package, and the previously solved A24-VYG structure (PDB = 2BCK) was used as a model for molecular replacement. Refinement and rebuilding was carried out using COOT and the data were scaled, reduced, and analyzed with the CCP4 package (PDB code: 414W).

### Results

**MHCI-restricted T-cell clones exhibit explicit peptide length recognition footprints**

To determine the extent of peptide length cross-reactivity displayed by TCRs, we examined the functional recognition of a custom-built "sizing scan" comprising random peptide libraries of different lengths (X9, X10, X11, X12, and X13), where X = any of the 19 L-amino acids excluding cysteine; Table 2) by a panel of CD8+ T-cell clones raised against peptides ranging from 9-13 residues (Figure 1 and supplemental Figure 1, available on the Blood Web site; see the Supplemental Materials link at the top of the online article). This MHCI-peptide length preference was not random, but reflected the length of the peptide sequence against which each clone had been raised. Accordingly, the ILA1, 1E6, SB14, SB10, and SB27 clones demonstrated a recognition preference for 9mer, 10mer, 11mer, 12mer, and 13mer peptides (Figure 1), respectively.

Although the sizing scans indicated that individual TCRs can exhibit an MHCI-peptide length preference, more detailed data can be obtained by performing positional scanning CPL scans across multiple peptide lengths. Therefore, we examined MHCI-peptide length preference by comparing the functional recognition of 8-13mer CPLs by 4 different CD8+ T-cell clones (ILA1, 1E6, MEL5, and SB27; Table 3). Figure 2A through D show, in heat map form, the detailed TCR recognition footprints of all 4 CD8+ T-cell clones (raw data are shown in supplemental Figures 2-5). ILA1, 1E6, peptide length (Figure 1 and supplemental Figure 1, available on the Blood Web site; see the Supplemental Materials link at the top of the online article). This MHCI-peptide length preference was not random, but reflected the length of the peptide sequence against which each clone had been raised. Accordingly, the ILA1, 1E6, SB14, SB10, and SB27 clones demonstrated a recognition preference for 9mer, 10mer, 11mer, 12mer, and 13mer peptides (Figure 1), respectively.

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### Table 2. Sizing scan parameters

<table>
<thead>
<tr>
<th>Sizing scan ID</th>
<th>Sequence of sizing scan mixture</th>
<th>Total no. of peptides in scan mixture (19°)</th>
<th>Concentration of each peptide in scan mixture*</th>
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<tr>
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<td>1.7 x 10^10</td>
<td>5.9 x 10^-15 M</td>
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<td>9mer</td>
<td>XXXXXXXX</td>
<td>3.2 x 10^11</td>
<td>3.1 x 10^-16 M</td>
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<td>10mer</td>
<td>XXXXXXXX</td>
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<td>1.6 x 10^-17 M</td>
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<td>12mer</td>
<td>XXXXXXXX</td>
<td>2.2 x 10^16</td>
<td>4.5 x 10^-20 M</td>
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<tr>
<td>13mer</td>
<td>XXXXXXXX</td>
<td>4.2 x 10^18</td>
<td>2.4 x 10^-22 M</td>
</tr>
</tbody>
</table>

n indicates the number of degenerate positions; *When mixtures are used at a concentration of 100μM.

**Table 3. CPL scan parameters**

<table>
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<tr>
<th>CPL ID</th>
<th>Sequence of position one sublibraries</th>
<th>Total no. of peptides in library (a + 19) x 19°</th>
<th>No. of sublibraries</th>
<th>No. of peptides in each sublibrary (19°)</th>
<th>Concentration of each peptide in sublibrary*</th>
</tr>
</thead>
<tbody>
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<td>1.1 x 10^-13 M</td>
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<td>OXXXXXXX</td>
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<td>180</td>
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<td>5.9 x 10^-15 M</td>
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<tr>
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<td>OXXXXXXX</td>
<td>9.4 x 10^12</td>
<td>200</td>
<td>3.2 x 10^11</td>
<td>3.1 x 10^-18 M</td>
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<td>OXXXXXXX</td>
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<td>220</td>
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<tr>
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<td>240</td>
<td>1.2 x 10^14</td>
<td>8.6 x 10^-18 M</td>
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<td>OXXXXXXX</td>
<td>7.1 x 10^18</td>
<td>260</td>
<td>2.2 x 10^15</td>
<td>4.5 x 10^-22 M</td>
</tr>
</tbody>
</table>

n indicates the number of degenerate positions; O, fixed sequence position (1 of the 20 proteogenic L-amino acids; O is moved systematically through the peptide backbone in a full CPL); X, degenerate position (1 of 19 proteogenic L-amino acids, excluding cysteine); and a, full peptide length.

*When mixtures are used at a concentration of 100μM.
MEL5, and SB27 exhibited a large number of responses to peptide mixtures from the corresponding length-matched CPLs, in accordance with the results obtained from the sizing scan analysis (Figure 1). In stark contrast, CPL scans of other lengths elicited a very low number of positive responses, with a large number of mixtures failing to elicit outputs above background levels (Figure 2A-D). Some functional responses could be detected at the nonpreferred peptide length for all CD8^+ H11001 T-cell clones, although it was not possible to identify responses at all peptide positions at the nonpreferred lengths (with the possible exception of the 8mer CPL scan on MEL5; Figure 2C). Therefore, detailed CPL scans of multiple CD8^+ T-cell clones suggest that every TCR is characterized by a defined MHCI-peptide length recognition footprint.

MHCI-peptide length preference is not determined by MHCI binding

HLA A24, B7, and B35 molecules are capable of binding peptides of different lengths (8-14 amino acids). However, limited data are available for the peptide length binding specificity of HLA A*0201 and this has not been assessed previously using unbiased peptide mixtures. Therefore, we examined the ability of HLA A*0201 to bind sizing scan mixtures of different lengths (Table 2). To accomplish this, we measured the peptide-dependent stability of HLA A*0201 on the surface of T2 cells by flow cytometry. These experiments demonstrated that HLA A*0201 is capable of binding to all sizing scan mixtures that span 8-13 amino acids in length (Figure 3 and supplemental Figure 6). Importantly, under conditions that were identical to those used in the sizing scan assays (Figure 1 and supplemental Figure 1), sizing scan mixtures of all lengths bound efficiently to cell-surface HLA A*0201 (Figure 3). In addition, levels of binding for all peptide lengths were similar to those observed for the immunodominant influenza A matrix protein (M1)-derived epitope GILGFVFTL (residues 58-66; Figure 3), which is known to bind strongly to HLA A*0201. These data suggest that the peptide length preference exhibited by each individual TCR is not determined by differential MHCI-peptide binding. Instead, the peptide length preference of individual CD8^+ T-cell clones appears to be determined strictly by the TCR.
Peptides designed from CPL scan data confirm MHCI-peptide length preference

Next, we designed peptide sequences based on the preferred amino acids at each peptide position as revealed by CPL scan analysis (Figure 2) for the CD8+ T-cell clones 1E6, MEL5, and SB27. Previously, we demonstrated that > 6 x 10^6 decameric peptides can act as substantially more potent agonists for the 1E6 TCR compared with the index peptide. Remarkably, in contrast, activation of 1E6 CD8+ T cells with a 9mer CPL scan yielded a very low number of positive responses relative to the 10mer CPL scan (Figure 2B). To study this observation in more detail, we designed panels of 9mer and 10mer peptides from combinations of the amino acids that elicited the strongest responses at each position observed in the corresponding CPL scans. The 9mer peptides tested on 1E6 were not recognized (Figure 4A-B). In contrast, the majority of 10mer peptides were recognized efficiently, with 13 of 17 acting as more potent agonists for this clone compared with the index peptide (Figure 4C-D). These data demonstrate that the 1E6 TCR exhibits a stringent preference for 10mer peptides compared with peptide sequences that are 9 amino acids in length, thereby verifying the CPL scan data (Figure 2B).
The MEL5 CD8+ T-cell clone also exhibited a strong preference for 10mer peptides in the corresponding CPL scan (Figure 2C). In addition, the best-recognized CPL sublibrary did not always correspond to the amino acid residue present in the index ELA-GIGILTV (ELA) peptide (Figure 2C). Multiple substitutions at position one (P1) of the index ELA peptide produced more potent agonists (Figure 5A), which is consistent with the structure of the TCR/pMHC complex33 (supplemental Figure 7). Here, the CDR1 loop of the MEL5 TCR lies closely over the N-terminus of the ELA peptide, offering opportunities for additional atomic contacts. A strong preference for P3-Thr and P8-Ile was also observed (Figure 5B). Single, double, and triple substitutions in the ELA peptide yielded a set of very strong agonists, the majority of which were considerably more potent than the index ELA peptide (Figure 5A-B). These results corroborate the CPL scan data demonstrating that a large number of 10mer peptides can be recognized efficiently by the MEL5 TCR. In contrast, 9mer and 11mer sequences designed from the low responses observed in the corresponding CPL scans exhibited poor activation potency, with the exception of one 9mer peptide, FWLLPAWAL, which activated MEL5 CD8+ T cells with intermediate potency (Figure 5C). pMHC1 tetramer staining with peptide variants was consistent with the activation data (Figure 5D). Therefore, the MEL5 TCR preferentially engages 10mer peptides.

It is now widely accepted that longer peptides play an important role in MHCI-restricted immunity.18,24,34 The CD8+ T-cell clone SB27, which recognizes the EBV BZFL1-derived 13mer peptide LPEPLPQGQLTAY (LPE), epitomizes this phenomenon. Activation of SB27 with 13mer CPL screen mixtures yielded a very large number of responses, higher than any other CPL scan performed in the present study (Figure 2D). All 13mer peptides in an extensive panel designed from the corresponding CPL scan data activated SB27 efficiently (Figure 6C-D). It was also clear from the CPL scan data that the SB27 TCR exhibited a strong preference for 13mer peptides (Figure 2D). We examined a panel of 9mer peptides designed from the corresponding CPL scan and tested them for recognition by SB27. As expected from preceding length recognition data, activation was poor and approximately 10,000-fold lower compared with the index 13mer peptide (Figure 6A-B). This result implies that TCRs specific for longer peptides are not capable of engaging shorter peptides efficiently. Immunity to longer peptides may therefore require mobilization of specific portions of the CD8+ T-cell compartment that exhibit appropriate length specificity.

**Peptide length has a profound effect on TCR degeneracy**

We next examined the impact of peptide length on the level of TCR degeneracy using a previously verified method called CPL-based importance sampling,7 which incorporates a sampling bias toward strong agonists as indicated by CPL scan data and thus allows an estimation of the number of peptides of a defined length that can be recognized by a given TCR on the basis of a sample of manageable size (in this case, 30 peptides). To correct the observed distribution for this bias, the observations are weighted by the reciprocal of the original sampling probability, giving estimates of numbers of peptides of a defined length that can be recognized at varying functional sensitivities. Figures 2 and 4 show that the 1E6 TCR demonstrates a strict preference for 10mer peptides. Indeed, when we previously performed CPL-based importance sampling using this 10mer scan, we found a large number of robustly recognized peptides, with more than 25% being recognized at higher functional sensitivity than the index peptide sequence.7 Responses to scan mixtures at all other peptide lengths were either entirely absent or very poor (Figure 2). To characterize the diminished efficacy of the interaction with 11mers and 12mers, CPL-based importance sampling was performed. In stark contrast to previous results obtained using 10mer CPL scan data, sampled 11mer peptides were all recognized with > 1000-fold lower efficiency compared with the index peptide (Figure 7A and supplemental Table 1). Similarly, sampled 12mer peptides were also poorly recognized by the 1E6 TCR (Figure 7B and supplemental Table 1).

Whereas CPL-based importance sampling performed at the preferred peptide length yielded small peptide libraries that were highly enriched with agonists, the extent of enrichment observed at the nonpreferred peptide lengths was lower, possibly because CPL scans performed at these peptide lengths contain less information. Alternatively, the degree of enrichment may be of comparable magnitude in all cases, but the occurrence rate of strong agonists (determined by both TCR degeneracy and degree of enrichment) remains too low to render agonists readily detectable in the samples. Degeneracy curves for the 1E6 TCR are shown in Figure 7C; the absissa is the functional sensitivity of peptides relative to the index peptide (ie, peptides with a value < 0.0, or > 0 are either poor agonists compared with index, equivalent to index, or better agonists than index, respectively), whereas the ordinate axis is the number of peptides that exhibit a functional sensitivity of at least the absissa. The degeneracy curves suggest that no 11mer or 12mer peptides were recognized with a functional sensitivity equivalent to index, indicating that peptide length has a major impact on the degeneracy profile of a TCR.

**Different length peptide agonists can occur but are often poorly recognized and distinct in sequence**

We have shown that Ag recognition by MHCI-restricted TCRs is constrained by peptide length, and that efficient recognition of peptides with alternative lengths is less likely. Interestingly, we identified one 12mer peptide that could be recognized by 1E6, although the efficiency of recognition was approximately 10-fold lower compared with the index peptide (Figure 7B). The sequence of this 12mer peptide (MMLWLRWIDVPT) was entirely different from the index sequence (ALWGPDPAAA) for the 1E6 TCR. Furthermore, 2 × 8mer epitopes and 1 × 9mer epitope were recognized by the MEL5 TCR (Figure 5C-F). Again, the sequences of these peptides (WLLPAWGV, WLLPTWGV, and FWLLPAWAL) were entirely different from the index sequence for the MEL5 TCR (ELAGIGILTV). Therefore, although rare, recognition of different length peptides is possible. However, the eliciting peptide sequence can be distinct from that of the index peptide and recognition typically occurs with reduced levels of functional sensitivity.

**Peptide length has a major impact on the complexity of antigenic structure**

To examine the underlying molecular mechanism of peptide length specificity exhibited by MHCI-restricted TCRs, we conducted a structural comparison of pMHC complexes recognized by the T cells used in this study. The atomic structure of the nonligated ELAGIGILTV (10mer),35 ALWGPDPAAA (10mer),36 HPVGEADY-FEY (11mer),37 CPSQEPMISYY (12mer),38 and LPEPLPQQQLTAY (13mer)43 peptides, in complex with their corresponding MHCIIs, have been solved previously. We also included previously solved structures of an 8mer39 and 14mer40 peptide in complex with MHCI (Figure 8). To complete our structural comparison of peptides
Figure 5. The MEL5 TCR exhibits a strict preference for 10mer peptides. C1R-HLA A*0201 cells (6 × 10^5) were pulsed with the indicated 10mer (A-B), 9mer (C), 11mer (D), or 8mer (E) peptides at the concentrations depicted for 2 hours at 37°C. Subsequently, 3 × 10^4 MEL5 CD8^+ T cells were added and incubated overnight. The supernatant was then harvested and assayed for MIP1β by ELISA. Error bars represent SDs. (D,F) MEL5 CD8^+ T cells (5 × 10^4) were incubated with PE-conjugated HLAA*0201 tetramer (25 μg/mL) folded around ELAGIGILTV (black), FWLLPAWAL (red), FWLLGAVAL (blue), FFAGGIGIRT (cyan), FLAGGIGIRTL (green), WLLPAWGV (yellow), or WLLPTWGV (pink) for 15 minutes at 37°C and then stained with 5 μL of 7-aminoactinomycin D for 30 minutes at 4°C, washed twice, and resuspended in PBS. Negative control staining is shown in dark purple.
corresponding to the CD8+ T-cell clones included in this study, we solved the atomic structure of HLA A*0201-ILAKFLHRL (9mer), recognized by ILA1, to a resolution of 1.8Å (supplemental Table 2). The overall structure of HLAA*0201-ILAKFLHRL was similar to previously solved pMHCI structures, with the peptide anchored at position 2 and the peptide C-terminus, enabling the central residues to bulge out away from the groove for potential TCR interactions. The analysis demonstrated that the positions of the anchor residues for each of the different length peptides were superimposable (Figure 8). Therefore, the distance between peptide residue 2, and the C-terminus of every peptide investigated, irrespective of differences in length, was very similar (approximately 20Å). Consequently, the only option available to accommodate extra residues as MHCI-bound peptides increase in length was an incremental increase in: (1) the degree of backbone “bulging” (ie, the height of the central peptide bulge away from MHCI groove; Figure 8A), (2) sideways displacement of the peptide toward the MHCI α1 or α2 helices (Figure 8B), or (3) a combination of both. For example, the central residues in the LPEPLPQQLTAY 13mer peptide bulged out of the groove by approximately 20Å, compared with only approximately 8Å for the ILAKFLHRL (9mer) peptide. The increasingly complex antigenic shape of the longer peptides revealed by this structural meta-analysis may restrict some TCRs from mediating stabilizing interactions with the MHCI surface, as described previously.24 This notion is further supported by our observation that individual TCRs are hard-wired to recognize peptides of a specific length and presumably cannot adapt to large structural variations in peptide conformation. Therefore, antigenic complexity (degree of peptide backbone “bulging”/sideways displacement) is determined by peptide length (in addition to peptide sequence), which in turn determines the outcome of TCR/peptide-MHCI engagement.

**Figure 6. Poor recognition of shorter peptides by the SB27 TCR.** T2-HLA B*3508 cells (6 × 10⁶) were pulsed with the indicated 9mer (A-B) or 13mer (C-D) peptides at the concentrations depicted for 2 hours at 37°C. Subsequently, 3 × 10⁶ SB27 CD8+ T cells were added and incubated overnight. The supernatant was then harvested and assayed for MIP1β by ELISA. Error bars represent SDs.
Discussion

Individual αβ-TCRs can recognize vast numbers of peptides encapsulated within a singular length. This inherent degeneracy is essential for effective immune coverage against the enormous number of antigenic peptides that could be encountered. Given the remarkable extent of such length-confined TCR plasticity, it seemed likely that TCRs could also cross-react with peptides of different lengths in the context of the same, or even different, presenting MHCI molecules. Indeed, the natural killer T-cell (NKT) TCR can recognize lipid Ags with various length head-groups, namely α-galcer (1 sugar) and iGb3 (3 sugars). Such peptide length flexibility would further maximize the ability of the numerically limited TCR repertoire to provide broad antigenic coverage. Indeed, the Ag processing/presentation machinery naturally generates MHCI-binding peptides across a range of fragment lengths, typically 8-14 residues. However, the degree to which αβ-TCRs cross-recognize these fragment lengths is ill-defined. In the present study, we used an approach that allowed for a sweeping and inclusive scan of TCR specificity. Surprisingly, we discovered a clear limit to receptor plasticity in terms of a defined peptide length preference. This unexpected finding was shown to be an inherent property of all TCRs examined across numerous biologic specificities and restricting MHCI alleles.

The data presented herein demonstrate that TCR plasticity is delimited by peptide length. Therefore, individual TCRs are predisposed to engage peptides of a defined length and cross-reactivity across peptide length boundaries is uncommon. These findings uncover a novel and fundamental feature of MHCI-restricted T-cell immunity that has wide implications. In particular, they suggest that the CD8+ T-cell repertoire is compartmentalized...
by length preference. Effective MHC-mediated immunity would therefore require the mobilization of length-matched Ag-specific CD8+ T-cell clonotypes from the peripheral repertoire. This is especially pertinent to the design of “superagonists” or heteroclitic ligands that are not amenable to a priori prediction on the basis of length or sequence.

To understand the molecular mechanism that underlies the MHC-peptide length preferences displayed by different TCRs, it is informative to consider the conformations adopted by MHC-bound peptides of different lengths. We show that incremental peptide length or sequence.

Figure 8. The degree of MHCI-peptide backbone “bulging” is proportional to peptide length. Supposition of pMHCI structures with increasing epitope length. The peptides have been superimposed on HLA A*0201, which is shown in cartoon representation. MHCIs were aligned using the MHC 2 helix (below) are shown. PDB codes are 1A1N, 10GA, 1A6P (grey), 12mer B*3508-HPVGEADAYFEY (blue), 12mer B*3508-CP5EQMPSIVYY (grey), 13mer B*3508-LPPLPGQGQLTAY (yellow), and 14mer B*3501-LPAVVGSPGEGFEV (purple). (A) Side view demonstrating the different peptide bulges out the MHCI groove depending on the length of the peptide. The scale on the left was calculated using the conserved position of peptide residue 2 from the Cx atom as 0 Å. The MHCI α2 helix has been removed for clarity. (B) “Bird’s eye view” demonstrating the different sideways displacement of peptides toward MHCI α1 or α2 helices depending on the length of the peptide. The scale on the left was calculated using the average central position of all of the peptides as 0 Å. The MHC α1 (above) and α2 helix (below) are shown. PDB codes are 1A1N, 10GA, 2GT9, 2FZ3, 3BW9, 12HL, 1XH3, and 414W.
showed no correlation with peptide length specificity. Further studies are required to define a role for TCR usage as a determinant of MHCI-peptide length preference.

In summary, we have shown that MHCI-restricted T cells exhibit a preference for peptide length that is governed by the TCR and that effective recognition of pMHCI Ag can only be achieved by length-matched Ag-specific CD8+ T-cell clonotypes. This novel finding has broad implications for understanding how effective CD8+ T-cell immunity is achieved and the future design of peptide vaccination. In addition, we propose that every TCR is characterized by a unique “peptide-recognition signature” that is defined by: (1) a preference for peptide length, (2) the number of peptides that can be recognized, and (3) the amino acid sequence of these agonist peptides. The peptide recognition signature of an induced CD8+ T-cell response is likely to have a major impact on CD8+ T-cell immunity and could underlie the pathogenesis of many disease states.

Acknowledgments
This work was supported by the Biotechnology and Biological Sciences Research Council (grant BB/H001085/1) and The Wellcome Trust (grant WT079848MA). L.W. is a Wellcome Trust Clinical Intermediate Fellow, D.A.P. is a National Research Council Senior Clinical Fellow, and D.K.C. is a Wellcome Trust Career Development Fellow. J.J.M., J.R., and S.R.B. are funded by the National Health and Medical Research Council. J.R. is a National Health and Medical Research Council Australia Fellow. A.S., G.D., M.P., and D.A.P. are funded by the Juvenile Diabetes Research Foundation (grants 7-2005-877, 1-2007-1803, and 17-2009-806). J.E.M., M.P.T., M.C., and T.W. are funded by The Wellcome Trust. Research by A.S. and M.P. is supported by the National Institute for Health Research Biomedical Research Center based at Guy’s and St Thomas’ National Health Service Foundation Trust and King’s College London. The views expressed are those of the author(s) and not necessarily those of the National Health Service, the National Institute for Health Research, or the Department of Health.

Authorship


Conflict-of-interest disclosure: The authors declare no competing financial interests.

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