Distinct Footprints of TCR Engagement with Highly Homologous Ligands

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Distinct Footprints of TCR Engagement with Highly Homologous Ligands

Fabio R. Santori,* Kaisa Holmberg,† David Ostrov,‡ Nicholas R. J. Gascoigne,† and Stanislav Vukmanovicć*§

T cell receptor engagement promotes proliferation, differentiation, survival, or death of T lymphocytes. The affinity/avidity of the TCR ligand and the maturation stage of the T cell are thought to be principal determinants of the outcome of TCR engagement. We demonstrate in this study that the same mouse TCR preferentially uses distinct residues of homologous peptides presented by the MHC molecules to promote specific cellular responses. The preference for distinct TCR contacts depends on neither the affinity/avidity of TCR engagement (except in the most extreme ranges), nor the maturity of engaged T cells. Thus, different portions of the TCR ligand appear capable of biasing T cells toward specific biological responses. These findings explain differences in functional versatility of TCR ligands, as well as anomalies in the relationship between affinity/avidity of the TCR for the peptide/MHC and cellular responses of T cells. The Journal of Immunology, 2004, 172: 7466–7475.

The biological response elicited by TCR ligands is dependent on the developmental stage of the T cell and the affinity/avidity of interaction (1, 2). Relatively high affinity/avidity TCR ligands induce cell death of immature CD4+/CD8+ thymocytes (negative selection), while proliferation, cytokine secretion, and/or cytolytic activity (agonist activity) are elicited in mature T cells. The low affinity/avidity TCR engagement promotes positive selection, i.e., survival and differentiation of immature thymocytes into mature CD4+CD8- or CD4+CD8+ T cells. In mature T cells, low affinity/avidity ligands may antigenize T cell responses induced by agonist ligands in vitro (3) and possibly in vivo (4), and can also promote survival and/or homeostatic expansion of naıve T cells (1). Although the spectrum of biological activities elicited by peptide/MHC usually correlates with the affinity for the TCR, numerous exceptions have been identified (5, www-ermm.cbcu.cam.ac.uk/01002502h.htm). For example, peptides with low affinity/avidity are capable of fully stimulating mature T cells (6–10). In addition, some of the same peptides that stimulate proliferation of mature T cells can also promote positive selection (11–15). The kinetics of the TCR engagement was proposed to be the principal determinant of stimulatory potential of TCR ligands (16). Although biological activity correlates better with the $t_{1/2}$ of peptide/MHC-TCR interaction than with the affinity/avidity of association (7), exceptions to this rule have also been noted (10).

Stimulation by anti-TCR Abs with specificities for TCRα or TCRβ chain had distinct effects on thymocytes, suggesting that physical engagement of TCRα may induce signaling events distinct from engagement of TCRβ (17, 18). Indeed, disposal of TCRα-connecting peptide motif was detrimental for positive (and not negative) selection, for extracellular signal-regulated kinase activation by the ligands that promote positive selection, and for recruitment of CD3ζ chain into TCR/CD3 complex (19). In contrast, deletion of a counter structure in TCRβ chain prevented negative, but not positive selection (20). Collectively, these findings may suggest that TCRα, as opposed to TCRβ, is uniquely equipped for generating signals leading to positive selection. This notion is further supported by selective usage of certain Vβ families in CD4+ or CD8+ T cells (21, 22). If this is true, then physiological ligands that induce positive vs negative selection should be expected to follow distinct rules of TCR engagement, focusing preferentially on contacting TCRα or TCRβ, respectively. Furthermore, similar differences could be expected in peripheral T cells because ligands that induce positive or negative selection can also initiate cellular responses in mature T cells. Different types of TCR engagements can be envisioned if ligands that promote distinct T cell responses are structurally different. However, in most experimental models, similarity to Ag is essential for biological activity of ligands that induce positive selection and antagonist activity (23–25). This similarity is especially important at the TCR contact residues in the C terminus of the peptide, which interacts with TCRβ (26–28). To assess whether different cellular responses of T cells require distinct TCR engagements, we performed comparative mapping and mutational analysis of three homologous peptides that promote distinct T cell responses in the H-Y-specific TCR transgenic model. Our results demonstrate that distinct rules of TCR engagement operate for different cellular responses of T cells, despite high degree of similarity between the specific ligands.

Materials and Methods

Peptide binding to MHC class I

Custom synthesized peptides were purchased from Research Genetics (Huntsville, AL). Peptide binding to and dissociation from H-2Dβ was...
verified by RMA-S stabilization assay. Briefly, 2 × 10^6 RMA-S cells were plated overnight at room temperature with different concentrations of peptide. Cells were then incubated at 37°C for 1 h, washed in PBS, and stained for 30 min with anti-H-2D^b Ab KH95 (BD PharMingen, San Diego, CA). Cells were then washed in PBS and stained for 30 min with secondary antibody for FITC or PE. After secondary staining, cells were washed and analyzed by FACS. For dissociation assays, we pulsed 1 × 10^6 RMA-S cells overnight at room temperature with 30 μM test peptide. Cells were then washed 1 × with PBS, and incubated for 0, 2, 4, or 6 h at 37°C. After each time point, cells were stained, as described above.

**Proliferation assay**

Spleen cells (1 × 10^6) from H-Y-specific TCR transgenic mice (purchased from Taconic Farms, Germantown, NY) were incubated with irradiated 5 × 10^6 spleen cells from C57BL/6 mice pulsed with a range of concentrations of the test peptide 100–0.001 μM agonist in round-bottom 96-well plates in RPMI 1640 medium supplemented with 5 × 10^{-5} M 2-ME, 1 mM sodium pyruvate (Life Technologies, Gaithersburg, MD), 0.1 mM nontoxic amino acids solution (Life Technologies), and 10% FCS (PM-10 medium). After 48–72 h, as indicated, each microculture was pulsed with 0.5 μCi of [3H]thymidine (ICN Biomedicals, Costa Mesa, CA) overnight, and thymidine incorporation was subsequently measured on a beta scintillation counter 1450 MicroBeta (Wallac, Turku, Finland). To test the antagonist activity of peptides, 5 × 10^6 irradiated splenocytes of C57BL/6 female mice were incubated for 1 h at 37°C with a suboptimal dose of the original Smcy 738,4 (100 nM). The cells were then washed once with PM-10 medium, and 5 × 10^5 cells were incubated with 1 × 10^5 H-Y-specific T responder cells in the continuous presence of indicated concentrations of peptides tested for antagonism (23).

**Coreceptor down-modulation dulling assay**

The assay was performed, as described (29). C57BL/6 mice were injected i.p. with 3 ml of thymocyte solution. After 3–4 days, mice were sacrificed and the activated macrophages were collected with cold PBS. The cells were washed, resuspended in PM medium supplemented with 20% FCS, and plated in flat-bottom 96-well plates at the density of 1.5 × 10^5 cells/well. Macrophages were incubated for at least 1 h at 37°C when they received 4 × 10^4 thymocytes/well derived from either male or female H-Y, B10.BR, or control C57BL/6 mice, with TAP1^−/− status. The cultures were then pulsed with the desired concentration of peptide and incubated overnight at 37°C. The next day, the thymocytes were collected and stained for FACS analysis with anti-mouse CD4 PE and CD8 CyChrome Abs (BD PharMingen). Dulling was quantified based on the shift from the gate of untreated double-positive (DP)^3 thymocytes from those of peptide-treated DP thymocytes. Peptide-treated thymocytes were also collected and tested for apoptosis using a FITC-based TUNEL assay kit (Boehringer Mannheim, Indianapolis, IN).

**Fetal thymus organ cultures (FTOC)**

The FTOCs were performed using gestation day 16 fetuses (23). Fetal thymus lobes were cultivated on sponge-supported cultures. As negative control, we used peptides that bind H-2Db well, but do not induce positive selection of H-Y thymocytes. After 10 days, lobes were dissociated and stained for FACS analysis with anti-mouse CD4 PE and CD8 CyChrome Abs (BD PharMingen). Dulling was quantified based on the shift from the gate of untreated DP thymocytes from those of peptide-treated DP thymocytes. Peptide-treated thymocytes were also collected and tested for apoptosis using a FITC-based TUNEL assay kit (Boehringer Mannheim, Indianapolis, IN).

**Molecular modeling**

The atomic coordinates for the peptide/H-2Db complex most similar to Ube1x509-517, ARX54-62, and Smcy738-746 provided the basis for molecular modeling and yielded indistinguishable results: INQ, which is the structure of SSVVGVWYL/H-2D^b and does not have a Brookhaven Protein Data Bank code (30). The latter structure contains two key anchor residues at positions 5 (N) and 9 (L). The coordinates for the peptide in the crystal structure of SVNL9 complexed to H-2Db were used to model the corresponding residues in Ube1x509-517, ARX54-62, and Smcy738-746 using the program O, version 7.1 (31). The atomic coordinates were subjected to 200 steps of conjugate gradient energy minimization using the program CNS (32). The molecular models of Ube1x509-517, ARX54-62, and Smcy738-746 were submitted to D^b and bound to D^b were displayed using the program SETOR (33). The peptides and residues 1–135 of the H-2Db H chain are displayed. The residues corresponding to the α2-helix (136–176) were omitted for clarity.

**Tetramer-binding and inhibition experiments**

H-Y^D^b containing a biotinylation sequence and human β2-microglobulin were expressed in Escherichia coli and refolded, as described (34–36). Briefly, inclusion bodies dissolved in 8 M urea were mixed with peptide using 4.5 mg of H chain, 1.5 mg of L chain, and 1 mg of peptide in the presence of 10 mM DTT (34, 36). The mixture was dialyzed against 4 L of 20 mM Tris, pH 8.0, 150 mM NaCl at 4°C for 48–72 h. Refolded pMHC complex was separated with a Superdex HR200 column (Pharmacia Biotech, Uppsala, Sweden). Biotinylation was performed using the BirA enzyme and reagents from Avidity L.L.C. (Denver, CO) (35, 36). For tetramerization, pMHC was mixed with streptavidin-PE (Molecular Probes, Eugene, OR) in a molar ratio of 4:1. Tetramers were separated with the Superdex HR200 column. Concentration of tetramer was determined by Bradford assay. C57BL/6 splenocytes were irradiated and pulsed with 50–100 μM Smcy738-746 for 1 h. After washing, APC were mixed with H-Y^D^b transgenic thymocytes and cultured in RPMI 1640 medium supplemented with IL-2. The cells were restimulated 10 days later and, thereafter, cells were restimulated every week. After several stimulations, only CD8^+ T cells were left in the culture. Cells were washed twice with FACS buffer (PBS, 2% FCS, 0.1% NaN_3) before staining with peptide/MHC tetramers. Tetramer-staining experiments were performed, as described (36). Briefly, for equilibrium binding, cells were stained with tetramer at concentrations from 0.1 to 600 nM together with anti-TCR C6i mAb (H57-597). Crystallography studies have demonstrated that this Ab does not interfere with binding of peptide/MHC complexes (37). For inhibition, we stained with Ube1x509-517-4B tetramer (20 nM) in the presence of unlabeled pMHC monomer (5 × 10^{-7} to 10^{-5}). Staining was for a minimum of 2 h at 4°C. Cells were washed twice and fixed with 1% formaldehyde in PBS before flow cytometry. T cells were gated based on anti-TCR C6i staining.

**Tetramer decay experiments**

Tetramer decay was performed largely as described (10, 36, 38). Lymphocytes were stained with tetramer (20–100 nM), washed twice with FACS buffer, and kept on ice until they were mixed with excess anti-H-2Db mAb 28-14-8s supernatant and incubated at room temperature, to allow tetramer dissociation. Although mAb 28-14-8s binds to the α3 domain of H-2D^b, it prevents both association and rebinding of H-2D^b tetramers to the P14 TCR (39) (K. Holmberg and N. R. J. Gascoigne, unpublished observations). After 0–30 min, cells were washed and fixed for flow cytometry. The natural logarithm of percentage of geometric mean fluorescence (GMF) at each time point (compared with 0 min) was plotted against time. The tetramer 1/t_½ was derived from the slope by t_½ = ln2/slope.

**Results**

**Biological responses of T cells require distinct TCR contacts**

The Smcy738-746 peptide (KCSNRQRQL) is an Ag for the H-Y TCR (40). Mature TCR transgenic CD8^+ cells proliferate (Fig. 1a), while immature CD4^+CD8^- thymocytes down-modulate CD4 and CD8 molecules (Fig. 1b) and undergo apoptosis (Fig. 1c) in response to this ligand. CD4/CD8 down-modulation can occur in response to ligands that induce positive selection (24, 41), and is therefore not a direct measure of cell death. Nevertheless, ligands that induce positive selection induce strong coreceptor down-modulation only if the signals are nonspecifically enhanced. Therefore, strong coreceptor down-modulation induced by a ligand alone is a good indication of negative selection. The self peptide Ube1x509-517 (KSNLNRQRFL) antagonizes Ag-induced CD8^- thymocyte proliferation (Fig. 1d) and promotes generation CD4^-CD8^- thymocytes (Fig. 1e). These CD4^-CD8^- thymocytes are mature both phenotypically (low levels of CD24; Fig. 1f) and functionally (capable of responding to Ag; see Fig. 5c). In contrast, putative self peptide ARX54-62 (VSNLNRQRFL) that differs from Ube1x509-517.
Substitution at p4, p6, and p7 completely abolished the capacity of Smcy738–746 to induce proliferation of CD8+ T cells (Fig. 2a) or negative selection of CD4+CD8+ thymocytes (Fig. 2b). Substitution at p8 had the same effect (Table I), but is not shown in the figure because subsequent experiments indicated that Smcy738–746/8A did not bind to H-2D\(^{b}\) (Table I). To address the issue of p8 as a possible TCR contact, we synthesized another variant peptide (Y8L) that did bind to H-2D\(^{b}\) (Table I). Inability of this variant to induce proliferation (Table I) suggests that p8, too, is a TCR contact residue. Two Smcy738–746 variants (R4A and Q7A) that lost the ability to induce T cell proliferation acquired the ability to induce TCR antagonist activity and promote positive selection (data not shown), a phenomenon observed in other experimental systems (11, 13, 43, 44). In contrast, Smcy738–746/8A had no biological activity (Table I), suggesting a crucial role for peptide p6 in engaging the H-Y-specific TCR. The most striking activity was that of Smcy738–746K1A, which was indistinguishable from the original peptide (Fig. 1a). Even though a subtle quantitative loss in activity could be ascribed to the mutation at p1, due to slightly better binding to the H-2D\(^{b}\) of the mutant peptide (Table I), the lack of a deeper impact of p1 came as a surprise. p1 was expected to be a relatively important TCR contact based on differences between biological activities of Ube1x509 and ARX54, that differ only at position 1 (p1) induces antagonist activity, but not positive selection (23). Ube1x509 and ARX54 were discovered using homology to the Ag (Smcy738–746)-based bioinformatics algorithm. All three peptides have classical anchors at p5 and p9, described as motif for peptide binding to H-2Db (42). To determine which of the remaining seven residues are potential TCR contacts for the H-Y TCR, 1 aa at a time of these three peptides was substituted by alanine, except for p5 and p9. Each peptide variant was tested for induction of following biological activities: agonist or antagonist activity in mature T cell proliferation assays; positive and negative selection of thymocytes.

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The impact of N-terminal peptide residues is not imposed by indirect structural effects

The difference in biological activity of peptides could result from conformational differences between peptides or structural modifications of H-2D\(^{b}\) itself when bound to different peptides. Therefore, we generated molecular models of peptide/MHC complexes based on structural data of available peptide/H-2D\(^{b}\) complexes. These models suggest that the side chains of K1 in Smcy\(_{738-746}\) and Ube1x\(_{509-517}\) are positioned in a very similar, if not identical manner, and that Smcy\(_{738-746}\), Ube1x\(_{509-517}\), and ARX\(_{54-62}\) have very similar overall conformation (Fig. 3). In addition, side chains of most of the peptide residues are exposed to solvent, suggesting that amino acid alterations in the peptide could be accommodated without significant perturbations of the overall structures. The predicted orientation of the side chains is in agreement with all of the structures of peptide/H-2D\(^{b}\) complexes solved to date. These models therefore suggest that distinct identity of peptide N-terminal residues neither induce gross differences in the overall peptide conformation, nor cause alterations of the MHC class I structure that would prevent interactions with the H-Y TCR.

We have also tested possible structural alterations in peptide/H-2D\(^{b}\) functionally. If R at p4 is a crucial TCR contact for induction of T cell proliferation (and negative selection), then Ube1x\(_{509-517}\)–M, whose side chain has similar volumes and positions of positive selection. Twenty-six percent of CD4\(^{+}\)/CD8\(^{+}\)thymocytes were found in the Ube1x\(_{509-517}\)K1M-treated, vs 13% found in control peptide-treated FTOCs (Fig. 4a), while the original peptide generated an average net gain of ~20% CD4\(^{+}\)CD8\(^{+}\) thymocytes (see Figs. 1e and 2c). Of the remaining replacements, the most illustrative is that of M, whose side chain has similar volumes and length as K. Even though Ube1x\(_{509-517}\)K1M bound to H-2D\(^{b}\) as well as the original peptide and exhibited antagonist activity in T cell proliferation assays (Table I), it was unable to induce positive selection (net increase of only 3% of CD4\(^{+}\)/CD8\(^{+}\) thymocytes) (Table I). Collectively, these data argue against direct or indirect interference of peptide p1 on interactions with the H-Y TCR.

Qualities of side chains at p1 that promote positive selection

To understand the role of side chains at the peptide N terminus during positive selection, we focused on p1 because both the Ag and the ligand involved in positive selection have an identical residue at p1 with a side chain predicted to protrude out of the peptide-binding cleft further away than side chains of residues at p2 and p3. We replaced K1 in Ube1x\(_{509-517}\) with 7 aa in a decreasing order of similarity to the original residue (R, H, N, M, A, G, and Y), as defined by the PAM250 matrix (45). Only one of these replacements (R) did not alter the ability of Ube1x\(_{509-517}\) to induce positive selection. Twenty-six percent of CD4\(^{+}\)CD8\(^{+}\) thymocytes were found in the Ube1x\(_{509-517}\)K1R-treated, vs 13% found in control peptide-treated FTOCs (Fig. 4a), while the original peptide generated an average net gain of ~20% CD4\(^{+}\)CD8\(^{+}\) thymocytes (Fig. 4a). These results suggest that the ability of side chain at p1 to form salt bridges is crucial for induction of positive selection. The fact that other amino acids were also unable to promote positive selection when placed at p1 suggests that length of the side chain at p1 is also important factor for...
induction of positive selection of H-Y-specific T cells by Ube1x509-517.

Compensatory contacts of p1 on T cell proliferation and negative selection are context dependent

Taken together, the results presented so far suggest differential impact of central or N-terminal peptide residues on cellular responses of T cells. The requirement for distinct TCR contacts appears independent of the maturation stage of T cells, but specific for biological responses. To address the latter possibility directly, we first evaluated potential effects of p1 on mature T cell proliferation and negative selection of thymocytes. Comparison of biological activities of Smcy738–746 and the variant with K1A replacement at a wide range of concentrations showed only minor quantitative effects of p1 in the context of optimal TCR contacts at p4, p6, p7, and p8 (Fig. 1a). Biological activities of Ube1x509-517e and ARX54-62AR (Fig. 4d) further suggest that the presence of contacts at p1 does not compensate for suboptimal contacts near the peptide C terminus (F at p8). To assess the role of p1 and p4 in a highly controlled manner, we designed a series of multiple position variants of Smcy738–746 with A fixed at p2 and p3 and interleaved contacts at p1 and p4. SmcyKAAR induced mildly stronger proliferation of CD8+ T cells than SmcyAAAR (Fig. 4c). The residue at p1 could also contribute when suboptimal context was provided at p4: SmcyKAAK and SmcyAAAK peptides showed a weak agonist and an antagonist activity, respectively (Table I). However, p1 had no in

Table 1. Summary of biological activities of variants of Smcy738–746, Ube1x509-517e, and ARX54–62 peptides

<table>
<thead>
<tr>
<th>No.</th>
<th>Mutation</th>
<th>aa Sequence</th>
<th>H2-D Binding</th>
<th>Activity (T cells)</th>
<th>Activity (thymocyte selection)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Assoc. t1/2</td>
<td>Agonist</td>
<td>Antagonist</td>
</tr>
<tr>
<td>1</td>
<td>SmcyY538-746</td>
<td>KCSRNQYL</td>
<td>(1) 5</td>
<td>(2) 4</td>
<td>100 (9)</td>
</tr>
<tr>
<td>2</td>
<td>-KIA</td>
<td>A------------</td>
<td>(0.1) 5</td>
<td>(2) 1</td>
<td>100 (4)</td>
</tr>
<tr>
<td>3</td>
<td>-C2A</td>
<td>A------------</td>
<td>(0.1) 5</td>
<td>(2) 1</td>
<td>100 (4)</td>
</tr>
<tr>
<td>4</td>
<td>-S3A</td>
<td>A------------</td>
<td>(0.1) 5</td>
<td>(2) 1</td>
<td>71 (11)</td>
</tr>
<tr>
<td>5</td>
<td>-R4A</td>
<td>A------------</td>
<td>(0.1) 5</td>
<td>(2) 1</td>
<td>24 (19)</td>
</tr>
<tr>
<td>6</td>
<td>-R6A</td>
<td>A------------</td>
<td>(1) 5</td>
<td>(2) 1</td>
<td>88 (7)</td>
</tr>
<tr>
<td>7</td>
<td>-Q7A</td>
<td>A------------</td>
<td>(0.1) 5</td>
<td>(2) 1</td>
<td>88 (7)</td>
</tr>
<tr>
<td>8</td>
<td>-Y8A</td>
<td>A------------</td>
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<td>(0) 1</td>
<td>(2) NT</td>
</tr>
<tr>
<td>9</td>
<td>-Y8L</td>
<td>L------------</td>
<td>(0) 1</td>
<td>(4) 2</td>
<td>(1) NT</td>
</tr>
<tr>
<td>10</td>
<td>-KIV</td>
<td>V------------</td>
<td>(1) 4</td>
<td>(2) 1</td>
<td>100 (2)</td>
</tr>
<tr>
<td>11</td>
<td>SmcyAAA</td>
<td>AAAANQQL</td>
<td>(1) 4</td>
<td>(2) 1</td>
<td>23 (9)</td>
</tr>
<tr>
<td>12</td>
<td>-A4K</td>
<td>K------------</td>
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<td>(1) 2</td>
<td>65 (8)</td>
</tr>
<tr>
<td>13</td>
<td>-A4R</td>
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<td>(2) 1</td>
<td>100 (4)</td>
</tr>
<tr>
<td>14</td>
<td>-A1K/A4K</td>
<td>K------------</td>
<td>(0.1) 1</td>
<td>(2) 1</td>
<td>16 (3)</td>
</tr>
<tr>
<td>15</td>
<td>-A1K/A4R</td>
<td>K------------</td>
<td>(0.1) 1</td>
<td>(2) 1</td>
<td>80 (4)</td>
</tr>
<tr>
<td>16</td>
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<td>(2) NT</td>
</tr>
<tr>
<td>17</td>
<td>-A1R/A4K</td>
<td>R------------</td>
<td>(0.1) 1</td>
<td>(4) 1</td>
<td>(2) NT</td>
</tr>
<tr>
<td>18</td>
<td>Ube1x509-517</td>
<td>KENLNPQFL</td>
<td>(0.1) 4</td>
<td>(4) 1</td>
<td>72 (10)</td>
</tr>
<tr>
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<td>-KIA</td>
<td>A------------</td>
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<td>(2) 1</td>
<td>57 (5)</td>
</tr>
<tr>
<td>20</td>
<td>-S2A</td>
<td>A------------</td>
<td>(10) 2</td>
<td>(4) 2</td>
<td>(1) 57</td>
</tr>
<tr>
<td>21</td>
<td>-N3A</td>
<td>A------------</td>
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<td>(4) 3</td>
<td>(1) 68</td>
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<tr>
<td>22</td>
<td>-L4A</td>
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<td>(1) 4</td>
<td>(1) 1</td>
<td>73 (8)</td>
</tr>
<tr>
<td>23</td>
<td>-R6A</td>
<td>A------------</td>
<td>(0.1) 1</td>
<td>(2) 1</td>
<td>17 (13)</td>
</tr>
<tr>
<td>24</td>
<td>-Q7A</td>
<td>A------------</td>
<td>(1) 1</td>
<td>(4) 1</td>
<td>(1) 10</td>
</tr>
<tr>
<td>25</td>
<td>-F8A</td>
<td>A------------</td>
<td>(1) 1</td>
<td>(4) 1</td>
<td>(1) 13</td>
</tr>
<tr>
<td>26</td>
<td>-KIM</td>
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a Association of peptide to H-2Dβ is expressed as the lowest peptide concentration (μM) that induced H-2Dβ on RMA-S cells at least 50% of the maximal values obtained with Smcy738–746 peptide. The t1/2 is expressed as the longest time interval, with >50% of initial MHC class I stabilization preserved. agonist activity is expressed as percentage of proliferation observed in the presence of 1 μM peptide relative to the proliferation obtained with the same concentration of Smcy738–746 peptide. Antagonist activity is expressed as a percentage of proliferation observed in the presence of 1 μM peptide relative to the proliferation obtained with Smcy738–746 peptide. Glutamyl thymocytes induced by all peptide concentrations tested was scored at least 50% of the maximal values obtained by 10 μM Glutamyl thymocytes. Shown are means and SDs obtained in several experiments (number indicated in the parentheses). Positive selection was determined by: 1) increase in the percentage of CD8+ thymocytes in FT ac cultured with the test peptide over that found in control peptide-treated FTAC, or 2) the ability of thymocytes isolated in FTAC to proliferate to Ag (Smcy738–746, 1 μM). An increase of >50% of CD4+ CD8+ thymocytes induced by Smcy738–746 Ag peptide was scored as agonist activity, while same increase induced only by low peptide concentrations (0.1 μM) was scored as antagonist activity. Negative selection was tested by: 1) coreceptor down-modulation assay; 2) deletion in FTOC; or 3) TUNEL assay following stimulation in suspension cultures. +, Designates efficient deletion at all peptide concentrations tested; +/-, indicates deletion only at high peptide concentrations (20 μM and more). NT = not tested. Numbers of performed experiments are indicated in parentheses, except for positive and negative selection, in which numbers for each experimental approach, as indicated above, are provided in a separate column (Materials and Methods).
SmcyKAAR, induced strong negative selection (Table I). R at p4 was partially tolerated when suboptimal TCR contacts were provided at the C terminus of the peptide as in ARX54–62L4R (F at p8), which was able to induce positive selection at low peptide concentration (Fig. 5, b and c). Ube1x509–517L4R was even more efficient at the induction of positive selection due to the presence of K at p1. Therefore, p4 can replace N-terminal contacts to induce positive selection only if the TCR contacts at the C terminus of the peptide or at p4 itself are weakened.

**Biological activity of peptide/MHC complexes does not correlate with the affinity/avidity of interaction with the TCR**

Preferential use of p1/p4 residues could be ascribed to the effect they might exert on the affinity/avidity of peptide/MHC-TCR interaction. According to the affinity/avidity paradigm, biological activities of peptides would predict following rank: Smcy738–746 ≈ Smcy738–746K1A > ARX54–62L4R ≈ Ube1x509–517L4R > Ube1x509–517 ≈ ARX54–62. To test this prediction experimentally, we generated soluble MHC tetramers and assessed their binding to living cells (10, 36, 38, 46). Smcy738–746 had the strongest binding of all peptides (Fig. 6a). However, we were unable to reach the saturation point of tetramer binding with this peptide, consistent with previous reports (47). Smcy738–746 also displayed longest $t_{1/2}$ and the most potent competition as a monomer (Fig. 6, b–d). The next strongest peptide in all aspects was Ube1x509–517, followed by Smcy738–746 followed by Smcy738–746K1A and ARX54–62L4R. The weakest binding, $t_{1/2}$, and inhibition values were obtained with Ube1x509–517 and ARX54–62L4R.

Comparison of these measurements with functional activities of peptides shows that affinity/avidity can only predict the biological...
activities of peptides with the most extreme values of the affinity/avidity scale. Thus, Smcy738–746, with the strongest binding promotes agonist activity and negative selection. Conversely, peptides with low affinity/avidity binding promote antagonist activity (ARX54–62) and positive selection (Ube1X509–517). However, biological activity of peptides in the intermediate range of affinity/avidity is independent of their affinity/avidity rank. Thus, Smcy738–746K1A, readily induced negative selection at wide range of concentrations, while Ube1X509–517 or ARX54–62 at low concentrations promoted positive selection. At the same low concentration, these three peptides clearly had strikingly different potential to delete CD4⁺CD8⁺ thymocytes in FTOC (Fig. 5, a and b). Likewise, Smcy738–746K1A more efficiently induced mature T cell proliferation than did Ube1X509–517 or ARX54–62 (Figs. 1a and 4b). Smcy738–746K1A engaged the H-Y TCR with an affinity/avidity lower than Ube1X509–517 or ARX54–62. The affinity/avidity theory would require the latter two peptides to induce stronger negative selection than the Smcy738–746K1A. Therefore, the preferential use of p1 for positive selection and of p4 for mature T cell responses and negative selection is not determined by the impact of p1 and p4 on the affinity/avidity of TCR engagement.

**Discussion**

Taken together, careful mapping of TCR contact residues in the H-Y TCR model shows that biological activity is distributed over p1, p2, p4, p6, p7, and p8. A core group of residues (p6–8) is required for all biological activities of MHC-bound peptide and is sufficient for TCR antagonist activity. One of the core TCR contacts (p6 in this case) represents the major contact because peptides with nonconserved mutations at this position are unable to elicit any biological response (Table I; Figs. 1d and 2). Besides the core, additional contacts are required to induce T cell responses other than antagonist activity. The central portion of the peptide (p4) is critical for T cell proliferation and negative selection, while the effects on positive selection are context dependent. Reciprocally, the N-terminal region (p1 and/or p2) is required for positive selection, but the induction of T cell proliferation and negative selection is context dependent. The functional importance of additional TCR contacts is asymmetric and cannot be explained by compensatory effects of secondary TCR contacts in the absence of primary ones (48). Hence, we call these contacts response preference TCR contacts.

A question of theoretical and practical importance is the general applicability of the present findings. All self-restricted peptide/MHC-TCR interactions resolved structurally to date follow the common docking mode, differing only by up to 35 degrees in orientation (26–28). This means that interactions between specific portions of the TCR with corresponding portions of the peptide/MHC complex are more or less comparable between different TCRs. In that respect, TCRα chain interacts with N terminus, whereas TCRβ chain interacts with C-terminal peptide portion. So, are core and response preference TCR contacts likely to operate in TCRs other than the one used in this study? Data on the vital importance of central and/or C-terminal peptide residues for agonist activity are readily available (11, 13, 48–51). However, the use of the same TCR contacts for other T cell responses is known only in few of these examples. Homology of C-terminal TCR contacts to Ag was shown to be the key quality of self peptides that promote positive selection of the OT-I TCR transgenic thymocytes. Nonconservative replacements at p6 and/or p7 in this model abrogated the activation of both mature T cells by the cognate Ag (50) and of immature thymocytes by two physiological self peptide/MHC complexes (24, 41). Thus, the major contact for the OT-I TCR is located close to the C terminus, as is the case for the H-Y TCR. However, loss of all biological activity in another model occurred by mutations at p4 (13). Thus, the major TCR contact is typically located in the central and/or C-terminal portion of the peptide. In the vicinity of the major contact are secondary TCR contacts (48), which could belong to the core set of residues, or could be agonist preference contacts.

Although the importance of contacts between the TCR and central peptide residues is well established, the functional role of N-terminal residues of peptide is less clear. Direct or indirect (through water molecules) contacts between the complementarity-determining region 1α (CDR1α) peptide p1 were seen in most of the structures of peptide/MHC-TCR complexes (26). Mutational analysis has shown that interactions of the CDR1α are important for the overall binding of the TCR to the peptide/MHC complex (52). For most TCRs, contacting p1 was not important to induce agonist activity, although some exceptions have been noted (11, 13). Our data presented in this work support possible use of p1 for agonist activity, but only when contact at p4 was suboptimal. Perhaps this is also the case with natural epitopes in the above experimental models. Mutations at p1 have enabled agonist ligands
to induce positive selection (43, 44). However, the role of p1 in these cases was most likely different from the one observed in this study. Altering p1 in epitopes reduced the affinity/avidity of peptide/MHC-TCR interaction (2), whereas alterations in our case led to an increase. Thus, the true test for our observations would include alanine-scan analysis of positively selecting peptides. This has only been performed in the OT-I TCR transgenic model, in which two such ligands are known. For one ligand, p1 did not appear essential for activation of immature thymocytes (24), while in the other, a peptide with a replacement at p1 did not bind MHC well, and was therefore not tested functionally (41). C-terminal contacts in the former self peptide were identical with the Ag (24), and therefore did not create suboptimal conditions required for the use of p1. The same occurred in the H-Y model when C-terminal contacts identical with the Ag allowed selection without the role for p1 (peptide SmcyAAA). Similar to the role of p2 in selection of the H-Y thymocytes, p2 was also partially involved in OT-I thymocyte activation by the latter self peptide, which has less conserved core TCR contacts (41). Even though the role of p1 in this ligand could not be tested, these data suggest that peptide N terminus can be involved in selection of the OT-I TCR too.

Thus, there may be at least two ways to induce positive selection by a single peptide: with or without using interactions with the N terminus of the peptide. Under physiological conditions, several peptide species collaboratively promote selection of a given TCR, and each peptide is essential for selection (53). Thus, even though some self peptides may not select in an N terminus-dependent manner, selection of any given TCR may likely depend on contacts with N terminus of one or more peptides involved in collective selection. Consistent with this notion are data suggesting the crucial role of CDR1α in positive selection (21, 22), as well as the positioning of MHC class I-binding anchor motifs that allow selective exposure of the N-terminal peptide to the TCR. Although the C-terminal anchor is usually the very last amino acid whose side chain is buried deep into the peptide-binding cleft, the N-terminal anchors are as a rule never at p1, but instead at p2 or p5 (42). As a result, the p1 side chain is usually exposed to solvent and therefore available for contact by the TCR. Our data and the existing literature, therefore, suggest that contacting the N terminus of the peptide is a requirement for selection of most TCRs, albeit not necessarily with all of the self peptides involved in selection.

The amino acid composition of the CDR regions may explain the unequal effect of individual response preference TCR contacts (p4 > p1) on the affinity/avidity of peptide/MHC-TCR interaction. Although ~50% of CDR1α residues represent those capable of forming alcohol-based hydrogen bonds such as Y, S, or T (54), CDR3 regions are richer in amino acids that form salt bridges (55). However, biological activity of peptides did not correlate with the affinity/avidity of peptide/MHC-TCR interaction, especially in the intermediate range (Fig. 7c). This was even the case when dissociation rates, which in general tend to correlate with biological

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**FIGURE 6.** Binding of H-2Db peptide/MHC ligands to H-Y T cells. Tetramers were prepared with H-2Db-peptide complexes and assayed for binding to restimulated HY transgenic T cells. a, Binding of pMHC tetramers. Binding is plotted as GMF against peptide/MHC concentration. Binding of Smcy738-746 was variable.

b, Dissociation kinetics for pMHC tetramer binding by monomeric peptide/MHC. Monomers of various H-2Dβ/peptide complexes were titrated into a binding experiment with 20 nM Ube1x509-517L4R fluorescent tetramer. The amount of monomer required to inhibit tetramer binding by 20% was determined and used to calculate the relative inhibitory efficacy for the different peptide/MHCs. c, Summary of normalized data from several experiments on dissociation kinetics and monomer inhibition. All data are normalized to the values obtained with Smcy738-746. Normalization was performed before determination of mean and SD to compensate for day-to-day variability between experiments. Actual values for Smcy738-746 were \( t_{1/2} = 6.07 \pm 2.10 \text{ min} \) and monomer inhibition of 20% tetramer binding \( = 7.58 \times 10^{-6} \pm 3.34 \times 10^{-6} \text{ M} \).
activities of peptides better than other parameters of TCR binding to peptide/MHC (7). Thus, Smcy738-746K1A that has a faster dissociation rate from the H-Y TCR than Ube1x509-517L4R or ARX54-62L4R is a stronger promoter of mature T cell proliferation and negative selection. Functional potentials of Ube1x509-517L4R and ARX54-62L4R themselves are also inconsistent with the affinity/avidity paradigm. Despite slightly better binding to the H-Y TCR, the former peptide was mildly more potent in inducing positive selection. Lack of strict correlation between the affinity/avidity of peptide/MHC-TCR interaction and the functional potential of TCR ligands has been described before on several occasions (5–10), but has not been adequately explained. In our model, the presence or the absence of the response preference TCR contacts in the context of stronger or weaker core TCR contacts explains the functional activity of peptides with intermediate affinity/avidity. The effect of response preference TCR contacts cannot, we believe, override an unfavorable extreme affinity/avidity (either low or high) between the TCR and peptide/MHC. Instead, it can act within the intermediate affinity/avidity range to provide specific functional information to the engaged T cell. This view agrees with a model in which each peptide/MHC complex has intrinsic efficacy (3), i.e., inherent bias to induce specific biological responses independent of the affinity/avidity of the TCR engagement. Intrinsic efficacy was used to explain why strong agonist peptides induce thymocyte positive selection in all dose ranges (56).

T cells during thymic development learn to blunt the impact of the most N-terminal portion of peptides that engage the TCR with low affinity/avidity. This finding can partly explain higher sensitivity of CD4+CD8- thymocytes than of mature T cells observed in other experimental models (57, 58), and most likely is a consequence of a new threshold set by the selecting ligand (59). One interesting aspect of developmentally regulated TCR plasticity is the underlying molecular mechanism(s). An attractive speculation may be inferred from the ability of Abs to adopt different binding configurations (60). These different configurations were not dependent on induced-fit mechanisms following binding to the ligand. Instead, different structural isomers were found to pre-exist in equilibrium. Similar pre-existing isomers of TCR have been proposed for TCR as well, of which only one would be competent for ligand binding (28). We suggest that at least two of the many structural TCR isomers are capable of ligand binding, one form more sensitive than the other to the N-terminal end of the peptide. Both forms may be represented in different ratios by mature and immature T cells, resulting in dissimilar sensitivities for TCR preference contact residues.

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**References**


