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The interaction between TCR and peptide-MHC (pMHC) complexes is crucial for the activation of T cells as well as for positive and negative selection in the thymus. The kinetics and affinity of this interaction and the densities of TCR and pMHC complexes on the cell surface are determining factors for different outcomes during thymic selection. In general, it is thought that agonist pMHC, which cause negative selection, have higher affinities and, in particular, slower off-rates than partial or weak agonists and antagonists, which cause positive selection. In this study, we have used pMHC tetramers to investigate the kinetics of TCR-pMHC interaction for agonist, weak agonist, and antagonist ligands of the anti-lymphocytic choriomeningitis virus P14 TCR. Kinetics determined on the cell surface may be biologically more relevant than methods using soluble proteins. We can distinguish between agonists and weak agonists or antagonists based on the half-life and the avidity of tetramer-TCR interaction. Furthermore, we show that a weak agonist self-peptide that positively selects P14 TCR+ thymocytes has a tetramer half-life and avidity only slightly weaker than strong agonists. We show that, in fact, it can act as quite a strong agonist, but that its poor ability to stabilize MHC causes it instead to have a weak agonist phenotype. The Journal of Immunology, 2003, 171: 2427–2434.

Mature T cells can be activated to differing extents by different peptide-MHC (pMHC) ligands: agonists cause the full range of T cell activation phenotypes, from activation of signaling cascades to cytokine secretion or target cell killing and to proliferation. Other altered peptide ligands (APLs) can be weak or partial agonists or antagonists. Partial agonists elicit only some of the features of T cell activation and antagonists can disable the signaling induced in response to an agonist ligand. The developmental fate of maturing T cells depends on the type of pMHC-generated signal encountered in the thymus. Several groups have used pMHCs added to fetal thymic organ culture (FTOC) to study how peptides affect differentiation, finding that strong agonists delete the CD4+8+ double-positive thymocytes (reviewed in (1, 2)). However, addition of certain weak agonist or antagonist ligands can result in positive selection, leading to the development of CD8+ single-positive cells (1, 2).

Much recent work has focused on how the T cell distinguishes these ligands. Structural analysis of TCRs binding related APLs did not reveal any significant differences in the TCR-pMHC interaction to explain agonist vs antagonist activation (3), but a recent study has identified a conformational change in TCRα structure during TCR-pMHC binding (4). This remains to be confirmed in other structures. Current models of TCR-pMHC interactions in relation to signaling include “kinetic proofreading” (5) and “serial triggering” models (6). Kinetic proofreading at its simplest explains signal strength as determined by the duration of TCR-pMHC interaction. Agonist pMHC ligands remain bound for long enough to activate the complete signaling cascade, resulting in, for example, proliferation and cytokine synthesis. Interaction of partial agonists or antagonists with TCR then have a shorter half-life, resulting in incomplete signaling. In contrast, serial triggering is based on the T cell integrating the number of TCRs that have interacted with ligand and predicts that there is an optimal binding window or “dwell-time”: too short binding does not activate TCRs, but too long an interaction slows dissociation of TCR from pMHC so that fewer TCRs are triggered (7). For the most part, TCR-pMHC binding kinetics have supported a kinetic proofreading model, with longer half-lives corresponding to higher T cell activation, although not without some anomalies (8–10 and reviewed in Ref. 11). The differences in affinity and half-life between agonists and antagonists are surprisingly small. It is also possible to find weak agonist ligands that activate through a gradual build-up of signals, culminating in full activation, but where the early points in the signaling cascade are not detectable (12). Similar models apply during thymic selection, where a long TCR-pMHC interaction leads to negative selection and shorter interactions give positive selection. This was found to be the case in the one TCR system where TCR-pMHC binding has been measured and where positive and negative selection in FTOC have been studied (8). Some recent evidence consistent with serial triggering has been presented, where longer half-lives of complexes resulted in lower T cell activation than complexes with intermediate half-lives (7). However, a study where TCR affinity was greatly increased by mutagenesis showed increased T cell reactivity (13). This result argues strongly against serial triggering.

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3 Abbreviations used in this paper: pMHC, peptide MHC; LCMV, lymphocytic choriomeningitis virus; APL, altered peptide ligand; FTOC, fetal thymic organ culture; SPR, surface plasmon resonance; GMF, geometric mean fluorescence.

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Contacts between TCR and MHC α helices are important in determining the overall architecture of the TCR-MHC interaction (14–16) and are reflected in the on-rate, whereas the contacts between peptide residues and TCR have more of an effect on the off-rate (17). Although TCR kinetics cannot be the only parameter determining T cell activation strength, they appear to be a very important factor for T cells to distinguish between ligands of different biological activity.

It is important to note that direct measurements of TCR-pMHC binding affinity and kinetics have used purified soluble monomeric TCR and pMHC molecules interacting in solution (albeit usually with one of the reactants tethered to a surface, as in surface plasmon resonance (SPR) measurements using Biacore). Thus, these analyses of TCR-pMHC binding have used membrane-free forms of TCR and pMHC, both of which are naturally cell surface integral membrane proteins. The αβ TCR exists as part of a complex containing the CD3 δε, γε, and ε dimers. The coreceptors CD4 and CD8 also have an important role in modulating or stabilizing TCR-pMHC interactions. Although their precise role during TCR binding to pMHC is unclear, CD4 and CD8 molecules are believed to bind MHC molecules and enhance T cell responses, and they may have a direct role in TCR-MHC complex formation (18–20).

In addition, variations in the level of coreceptors during thymocyte selection can change positive to negative selection (21, 22). Hence, a truly biologically relevant affinity determination may require intact TCR-CD3 complexes, including coreceptors, and perhaps other components.

Tetramers of pMHC complexes have been used to define Ag-specific T cell populations (23–25). Tetramer staining can differentiate even closely related T cell clones by binding with higher or lower intensities, and the level of tetramer binding and its half-life have been shown to correlate with monomeric affinity and half-life, respectively (24, 26). These measures can therefore be used as surrogates for the monomeric values and may indeed have more validity because binding to TCR is measured in a noninvasive manner, on the surface of the T cell, in the presence of CD3, coreceptors, and other molecules. In this study, we compare several peptides using the P14 TCR system that recognizes a peptide coreceptors, and other molecules. In this study, we compare several peptides using the P14 TCR system that recognizes a peptide from the lymphocytic choriomeningitis virus (LCMV) glycoprotein (p33) presented on mouse H-2Db. We determine their binding avidities and half-lives as multimers. We show a good correlation between these results and previously reported characteristics of each peptide as an agonist, weak agonist, or antagonist and either positive or negative selector (27–33). In most cases, the negative selecting peptides have a longer half-life and higher avidity than the positive selectors. Most interesting is the apparently anomalous binding of a positive selecting/weak agonist peptide (29, 32, 33), which has a strong interaction with TCR, but a weak one with Dα, resulting in its biological phenotype.

Materials and Methods

Mice

P14-transgenic mice (B6.D2gen(TCR,CMV)32) specific for the p33 peptide of LCMV (34) were bred and maintained at the animal facility of The Scripps Research Institute (La Jolla, CA) in accordance with institutional guidelines.

Peptides and Abs

Peptides p33, A3V, S7A, A4Y, L6F, W4Y, G4Y, mDBM, and AV (Fig. 1A) were generated at the Amgen Institute (Boulder, CO) and purified by HPLC. TCR α cells were detected with FITC-conjugated TCRβ chain-specific mAb H57-597, while anti-Vα2-FTTC and anti-Vβ8-PE were used to detect transgenic T cells. CD4-PerCP, CD8-allophycocyanin, and CD69-PE were used in some experiments. H-2Dα-FTTC (KB95) was used in the RMA-S stability assay to detect cell surface H-2Dα. All mAbs were purchased from BD Pharmingen (San Diego, CA). Cells producing mAb 28-14-8s (anti-Dα) were obtained from American Type Culture Collection (Manassas, VA).

Preparation of pMHC tetramers

H-2Db H chain containing a biotinylated sequence (25) and human β2-microglobulin L chain were individually produced in Escherichia coli and refolded as described previously (35). Inclusion bodies were dissolved in 8 M urea and mixed with peptide using 4.5 mg H chain:1.5 mg L1 mg peptide in the presence of 10 mM DTT. The refolding was performed by dialysis against 4 L of buffer (20 mM Tris (pH 8.0) and 150 mM NaCl) at 4°C over 48–72 h. Refolded pMHC was purified by size exclusion chromatography using a Superdex HR200 column (Pharmacia Biotech, Uppsala, Sweden) concentrated with a Centriprep concentrator (cutoff 10 kDa: Amicon, Beverly, MA) to 0.5–1 ml for biotinylation. The biotinylation was performed using the BirA enzyme and reagents from Avvidity LLC (Denver, CO) (23). Free biotin and degraded protein were separated by a second run through the HR200 column. For tetramerization, pMHC was mixed with streptavidin-PE (Molecular Probes, Eugene, OR) in a molar ratio of 4:1. The streptavidin-PE was added over several hours to ensure an excess of pMHC during the process. Tetramers were separated by a third round of size exclusion chromatography. The concentration of tetramer was determined by the Bradford assay against a BSA standard curve.

Tetramer binding and inhibition experiments

Single-cell suspensions from spleens of P14-transgenic mice were prepared. RBC were lysed, and lymphocytes were washed twice with FACS buffer (PBS, 2% FCS, and 0.1% NaN3) before staining with pMHC tetramers. For equilibrium binding, cells were stained with tetramer at concentrations from 0.1 to 500 nM along with anti-TCR HB7 mAb (H57-597). Note that this mAb does not interfere with the pMHC binding site (36). For inhibition, we stained with p33 tetramer (1–4 nM) along with unlabeled pMHC monomer (10–3–10–10 M), which competes with the tetramer for TCR binding (37). Staining was performed on 2 × 104 cells. Cells were washed twice and fixed with 1% formaldehyde in PBS before flow cytometry. In all experiments, we gated on lymphocytes based on forward and side scatter and on T cells based on anti-TCRβ staining. The analysis was performed with CellQuest software (BD Biosciences, Redwood City, CA). Equilibrium binding data were plotted as a Scatchard plot of geometric mean fluorescence (GMF)/concentration of tetramer used in staining against GMF. GMF represents the amount of bound tetramer and concentration used for staining represents the amount of free tetramer. The avidity, Ks, is derived from the slope by Ks = 1/slope.

Tetramer decay experiments

Tetramer decay was performed largely as described elsewhere (24). Spleen cells were stained with tetramer (1–25 nM), as in equilibrium binding experiments above. Cells were washed twice with FACS buffer and kept on ice until they were mixed with excess anti-Dα mAb 28-14-8s supernatant and then incubated at room temperature to allow for tetramer dissociation. The anti-Dα mAb was used to block rebinding of tetramer to the TCR (24). Dissociation was followed for 0–180 min, whereas cells were washed quickly with ice-cold buffer to remove all unbound tetramer and blocking mAb and were fixed for flow cytometry. The natural logarithm of percentage of GMF at each time point (compared with 0 min) was plotted against time. The half-life of each pMHC multimer was derived from the slope by τ1/2 = ln2/slope.

MHC stabilization by peptide binding

RMA-S cells (5 × 103/well) were incubated overnight at 29°C and pulsed with peptides (10–7–10–10 M) for 30 min at 29°C. The cells were then incubated at 37°C for 3 h, washed once, and stained for Dα expression. Data are plotted as percentage of Dα staining compared with maximal staining with the best stabilizer (L6F) against peptide concentration.

TCR down-regulation assay

Spleen cells from a P14-TCR-transgenic mouse (5 × 103/well) were incubated at 37°C in RPMI 1640 (10% FCS) with EL-4 cells (103/well) along with varying concentrations (10–7–10–10 M) of peptides. After a 3-h incubation, cells were stained for CD4, CD8, and Vα2. CD8+ splenocytes were analyzed for TCR down-regulation by flow cytometry. Data were

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plotted as percentage of V\textsubscript{2} staining compared with unstimulated sample against concentration of peptide. The peptide dose resulting in 50% down-regulation of TCR (SD\textsubscript{50}) was then determined.

TCR down-regulation and CD69 up-regulation by different peptides normalized for cell surface pMHC concentration

RMA-S cells (10\textsuperscript{5}) were loaded with concentrations of different peptides that give stable cell surface D\textsubscript{b} expression comparable to that obtained with 10\textsuperscript{3} M mDBM. Loading was controlled by staining with anti-H-2D\textsubscript{b}-FITC in a separate tube. Peptide-loaded cells were mixed with 5\textsuperscript{10}\textsuperscript{5} P14-transgenic splenocytes and incubated at 37 o C for 3 h. After incubation, cells were stained for CD4, CD8, and V\textsubscript{2} or CD69 and examined by flow cytometry. The TCR down-regulation, data are shown as a percentage of V\textsubscript{2} expression remaining on the surface of CD8 cells compared with control where cells were incubated with RMA-S cells without peptide. For CD69 up-regulation, the percentage of cells expressing CD69 high is presented. The gate defining CD69 low vs CD69 high was set at 70 fluorescence intensity units.

**FIGURE 1.** Dissociation of pMHC tetramers from P14 T cells.  
A, Sequence of p33 peptide is shown with arrows pointing upward showing TCR contact residues and arrows downward showing MHC contact residues. The TCR contact arrows are proportional in height to the percentage of the accessible surface of the peptide that they represent (38). Mutated residues of each peptide used in the study are shown. mDBM is a natural P14-binding peptide isolated from the adrenal medulla. AV is an adenovirus peptide that does not bind P14 TCR and is used as a negative control.  
B, Dissociation of p33 and A4Y from P14-transgenic splenocytes. Cells were stained with p33 or A4Y tetramer and left to dissociate in the presence of anti-H-2D\textsubscript{b} mAb that blocks rebinding of tetramer. TCR\textsuperscript{+} cells are shown. Numbers above each plot show the time (minutes) each sample was allowed to dissociate.  
C, Dissociation curves of tetramers. The high avidity tetramers are shown on the left and lower avidity ligands on the right. Natural log of percentage of GMF at each time point compared with t = 0 is plotted against time. Half-lives are calculated from \( t_{1/2} = \ln 2/\text{slope} \) and are shown in the inset. A representative experiment of three to four performed for each APL tetramer is shown.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Mature T Cell Activation</th>
<th>Negative Selection (M)</th>
<th>RMA-S Stabilization (50%, ( \mu \text{M} ))</th>
<th>TCR Down-Regulation (%)</th>
<th>Tetramer Avidity (pM)</th>
<th>Tetramer ( t_{1/2} ) (min)</th>
<th>pMHC Monomer Inhibition</th>
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<td>Agonist\textsuperscript{a}</td>
<td>10\textsuperscript{-12}\textsubscript{a}</td>
<td>10\textsuperscript{-6}\textsubscript{a}</td>
<td>3.8</td>
<td>81.6</td>
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<td>S7A</td>
<td>Agonist\textsuperscript{b}</td>
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<td>10\textsuperscript{-6}\textsubscript{b}</td>
<td>1.3</td>
<td>70.0</td>
<td>524 ± 137</td>
<td>58.6 ± 1.2</td>
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<td>10\textsuperscript{-6}\textsubscript{c}</td>
<td>51</td>
<td>71.0</td>
<td>588 ± 92</td>
<td>174 ± 3.5</td>
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<td>Weak agonist\textsuperscript{d}</td>
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<td>ND</td>
<td>1.5</td>
<td>11.6</td>
<td>814 ± 146</td>
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<td>Weak agonist\textsuperscript{e}</td>
<td>10\textsuperscript{-7}\textsubscript{e}</td>
<td>10\textsuperscript{-3}\textsubscript{e}</td>
<td>0.97</td>
<td>16.0</td>
<td>783 ± 150</td>
<td>10.0 ± 0.8</td>
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<tr>
<td>W4Y</td>
<td>Very weak agonist\textsuperscript{f}</td>
<td>10\textsuperscript{-7}\textsubscript{f}</td>
<td>ND</td>
<td>4.8</td>
<td>14.2</td>
<td>990 ± 116</td>
<td>17.7 ± 3.4</td>
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<tr>
<td>mDBM</td>
<td>Weak agonist/antagonist\textsuperscript{g,h}</td>
<td>10\textsuperscript{-4}\textsubscript{g,h}</td>
<td>None\textsuperscript{e}</td>
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<td>Antagonist\textsuperscript{i}</td>
<td>None\textsuperscript{e}</td>
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<td>1.7</td>
<td>10.4</td>
<td>17,500 ± 3,570</td>
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<td>None\textsuperscript{a,j}</td>
<td>None\textsuperscript{a,j}</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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</tr>
</tbody>
</table>

Data on T cell activation and thymocyte selection is from the references shown: \textsuperscript{a} (27), \textsuperscript{b} (31), \textsuperscript{c} (30), \textsuperscript{d} (28), \textsuperscript{e} P. S. Ohashi, unpublished data, \textsuperscript{f} (33), \textsuperscript{g} (29), and \textsuperscript{h} (32). RMA-S stabilization data are derived from Fig. 5, TCR down-regulation from Fig. 6, avidity from Fig. 2, half-life from Fig. 1, and competition from Fig. 3.
Results

Strong agonist pMHC tetramers have longer half-lives for binding cell surface TCR than weak agonists or antagonists

The off-rate or half-life of the TCR-pMHC complex is related to the strength of signaling (8–10). The rate of dissociation of pMHC tetramers (“tetramer decay”) from T cells is related to the off-rate of the TCR-pMHC interaction (24). We therefore compared binding of different APLs as pMHC complexes to the P14 TCR (specific for the p33 peptide of LCMV glycoprotein bound to mouse H-2D<sup>B</sup>) on P14-transgenic cells by measuring the dissociation rate using flow cytometry. We stained P14 TCR-transgenic splenocytes with a relatively low concentration of pMHC tetramer (1–5 nM). With the antagonist G4Y tetramer, we had to use a higher concentration (20 nM) because otherwise the signal was undetectable by the first time point. The dissociation was performed at room temperature in the presence of anti-D<sup>B</sup> Ab to block rebinding (24).

The observed half-lives (Table I and Fig. 1C) clearly separate these APLs into two groups. A3V, p33, and S7A have comparatively long half-lives (173.6–58.6 min). These pMHC ligands have all been characterized as strong agonists, causing negative selection in FTOC (27, 28, 30, 31). The ligands that have been characterized as weak agonists (W4Y, L6F, A4Y) (28, 30, 31) and the antagonist G4Y (29) have much shorter half-lives (17.7–6.2 min; Table I and Fig. 1B and C).

A surprising result was the long half-life, 85.8 min, of mDBM, a natural P14-binding peptide (from dopamine/H9252-monooxygenase, an enzyme expressed in the adrenal medulla), which has been shown to be a partial agonist/antagonist of P14 T cells (29, 32) and a positive selector for P14-transgenic thymocytes in FTOC (33). The half-life of mDBM was more similar to the agonists than to the weak agonist/antagonist group. As shown below, mDBM tetramer and monomer binding consistently grouped this peptide with the strong agonist peptides.

Relative avidity of pMHC tetramers determined by equilibrium binding distinguishes agonists from antagonist

The intensity of staining at a particular concentration of each tetramer grouped these APLs into two clear sets, higher and lower binders, with very little variation within each group (Fig. 2A). To determine the avidities of different APLs for the P14 TCR, we stained P14-transgenic splenocytes with a wide range of concentrations of each tetramer. Avidity was determined from Scatchard analysis (Fig. 2B). The negative selecting peptides p33, S7A, and A3V had avidities in a very close range (\(K_D = 421–588\) pM; Table I). The positive selectors A4Y, L6F, and W4Y were in a weaker binding range (\(K_D = 783–990\) pM), and the antagonist G4Y bound even more weakly (\(K_D = 17.5\) nM). The negative control tetramer AV did not bind T cells and thus we could not determine its avidity (data not shown). Again, mDBM behaved more like a strong agonist or a negative selecting peptide than a weak or partial agonist, binding to P14 TCR efficiently even at a low concentration (\(K_D = 697\) pM). A3V was expected to have the strongest avidity because it had the longest \(t_{1/2}\) and its maximal staining intensity was equal to or higher than that of p33, but in all experiments p33 and S7A had slightly higher avidity. Although the differences in \(K_D\) values are small, they are still statistically different from p33 (\(t\) test, \(p = 0.02–0.00007\) except for S7A (\(p = 0.2\)). SPR measurement of equilibrium affinity for P14 TCR binding was reported as 6 µM for p33 and 35 µM for A4Y (38). It is noteworthy that the affinity differences seen with SPR for other series of related APLs were also in a tight range (8, 11).

**FIGURE 2.** Avidity of tetramer binding to P14 T cells. A, P14-transgenic splenocytes were stained with a 2 nM concentration of high-avidity tetramers or 4 nM of low-avidity tetramers. TCR<sup>+</sup> cells are shown. GMF of the histogram is shown on the upper right corner. B, Scatchard plot showing all tetramers. GMF/concentration is plotted against GMF and the \(K_D = 1/slope. K_D\) for each tetramer is shown in the inset. A representative example of three to five experiments is shown.
Inhibition of tetramer binding by competition with monomer pMHC

Because the avidity of tetramers was generally high and the differences between their relative avidities were too small to clearly separate strong and weak agonists, we set up another type of experiment, where APLs were compared as monomers rather than as multimers. We stained T cells with p33-D\(^\beta\) tetramer and at the same time inhibited the staining with different concentrations of unlabeled monomeric pMHC (37). The percentage of inhibition of tetramer staining compared with staining without competitor was plotted against a concentration of monomer pMHC (Fig. 3). Regardless of the tetramer used during competition, this experiment divided APLs clearly into two groups. The weaker ligands (A4Y, W4Y, L6F, and G4Y) inhibited p33 binding only very modestly, even at the highest concentration used (10\(^{-5}\) M). When we arranged these peptides into order based on their ability to inhibit tetramer binding, the order from strongest competitor to weakest was A3V > p33 > mDBM > S7A > A4Y, L6F, W4Y > G4Y > AV. This order is based on the average of four experiments. The concentration (normalized to p33) of monomer pMHC that was able to inhibit p33 tetramer staining by 20% is shown in Table I. Even in this experiment, which compared affinities of monomer rather than of multimer pMHC, the natural positive selector mDBM was among the strong binders. In contrast, S7A had a lower ability to compete with the p33 tetramer than did p33 or A3V, indicating lower affinity.

T cell activation and TCR down-regulation

The peptide mDBM was shown to be a weak agonist in experiments where equal peptide concentrations were compared for their ability to stimulate T cells (29, 32). Its relative strength in TCR binding led us to suspect that its weak activity in bioassays could be due to a weak ability to bind MHC. We determined the amount of each peptide required to give 50% down-modulation of TCR from the cell surface (SD\(_{50}\)). This was plotted against the relative ability of pMHC monomer to inhibit tetramer binding or against tetramer half-life. Fig. 4 demonstrates that mDBM had a much lower ability to stimulate T cells on the basis of peptide concentration (SD\(_{50}\)) than would be predicted from the ability of its complex with D\(^\beta\) to interact with the P14 TCR. We then performed an RMA-S D\(^\beta\) stability assay with mDBM and other peptides. RMA-S cells do not present any endogenous peptides due to TAP transporter deficiency and thus do not express stable MHC class I molecules on the cell surface, unless loaded with exogenously added peptides. This assay confirmed that mDBM binds very poorly to D\(^\beta\) compared with other peptides, including strong and weak agonists and antagonists (Fig. 5). This experiment also showed that A3V stabilized D\(^\beta\) less well than p33 or S7A, which

![FIGURE 3. Inhibition of tetramer binding with unlabeled pMHC monomers. P14-transgenic splenocytes were stained with 2 nM p33 tetramer and with varying concentrations of competing pMHC monomers. TCR\(^\gamma\) cells were gated for analysis. Percentage of inhibition of p33 tetramer staining in each sample compared with staining without competitor pMHC is plotted against concentration of competitor pMHC. A representative example of three experiments is shown.](http://www.jimmunol.org/)

![FIGURE 4. TCR-pMHC binding strength vs peptide SD\(_{50}\) (concentration of peptide required to give 50% down-modulation of TCR). P14-transgenic splenocytes were stimulated with each peptide for 3 h. TCR down-modulation on CD8 single-positive cells was determined by flow cytometry and the SD\(_{50}\) for each peptide was calculated. A, Relative inhibition of p33-D\(^\beta\) tetramer binding by various pMHC monomers plotted against SD\(_{50}\). B, Half-life of D\(^\beta\) tetramers with various peptides plotted against SD\(_{50}\).](http://www.jimmunol.org/)

![FIGURE 5. RMA-S MHC class I stabilization assay. RMA-S cells were loaded with peptides at 29\(^\circ\)C and transferred to 37\(^\circ\)C to allow unstabilized MHC to degrade. Cells were then stained with anti-H-2D\(^\beta\) mAb and analyzed by flow cytometry. Data are shown as percentage of stabilization compared with maximal stabilization with L6F plotted against concentration of peptide.](http://www.jimmunol.org/)
might explain its unusual titration in T cell activation assays (see Discussion) (30).

To demonstrate the actual responsiveness of T cells to each ligand, we normalized the number of peptide-MHC complexes presented to the T cells by using RMA-S cells. We determined the concentration of each peptide that gave equal cell surface D\(^{b}\) expression on the RMA-S cells as the maximal loading with mDBM (1 mM; Fig. 6A). We used these cells to analyze TCR V\(_{α} 2\) down-regulation or CD69 up-regulation on P14-transgenic splenocytes (Fig. 6, B and C). In these assays, the response to each peptide was comparable to its relative tetramer binding. Therefore, this further demonstrates that mDBM in fact has a relatively high affinity for TCR and that its poor ability to stimulate T cells is related to its presentation on class I.

**Discussion**

We have used MHC class I tetramers with APLs of different biological activity for stimulation of the P14 anti-LCMV to analyze the relationship among off-rate, affinity, and the biological response. The avidity of tetramer binding gives relative values that can be used as a surrogate for true affinity measurements, such as SPR analysis (26). Off-rate measurements for monomeric TCR-pMHC interactions can be substituted for by measuring the half-life of tetramers bound to TCR (24). Tetramer-binding analysis has the advantage of measuring the interactions in the natural habitat of the TCR, i.e., on a cell surface in association with CD3 elements, coreceptors, etc., but the disadvantage that these are not direct measures of affinity or rate constants between pure molecules.

Each APL used in these experiments has been tested in earlier studies in FTOCs and determined to be either a positive or a negative selector (28, 30, 31). Similarly, the negative selectors were shown to be strong agonists for mature T cells, whereas the positive selectors were weak agonists and antagonists (28, 30, 31). In this work, each of the three types of tetramer-binding experiments showed that tetramers with peptides that are known to cause negative selection (strong agonists: p33, A3V, and S7A) had, as a group, a higher avidity and longer \(t_{1/2}\) than those that cause positive selection (weak agonists: L6F, A4Y, and W4Y) or the non-selecting antagonist G4Y. However, the rank order within these groups did not fit exactly with the reported biological potency of the ligands. In this regard, the avidities determined by direct tetramer binding did not show large \(K_{D}\) differences even between strong and weak agonists, suggesting that this is the least useful method for comparison of characteristics between different APLs. Half-life and the capacity of monomer pMHC to compete for tetramer binding were better measures.

Our results are broadly consistent with the kinetic proofreading model (5), as the stronger ligands have longer half-lives and weaker ligands much shorter half-lives. An apparent exception to the half-life: activity correlation is the peptide A3V. This has a longer half-life and a higher affinity (as determined by competition) than the other agonist ligands, but a lower avidity (as determined by tetramer equilibrium binding). It was previously shown to be able to activate T cells at high concentration but not at lower concentration (30). However, in our peptide titration experiments (Fig. 4 and data not shown), it down-regulated TCR and activated well at first (3 h data), but after sustained stimulation (16 h) the activation dropped below that of p33 or S7A, independent of concentration. A similar trend was seen previously in proliferation experiments (30). This may be caused by a faster dissociation of A3V from MHC. On thymocytes, a high concentration of A3V caused negative selection similar to p33 and S7A (30). The crystal structure of p33-D\(^{b}\) showed that the third position valine is buried in the MHC and is not available for TCR binding (38), and our
RMA-S stability assay showed that A3V was poor at stabilizing D\textsuperscript{b}. Our experiments with stimulation with equivalent numbers of D\textsuperscript{b}-peptide complexes showed that it is similar in efficacy to p33. These results suggest that the A3V-D\textsuperscript{b} complex has a strong interaction with TCR and is a strong agonist, but that it appears in peptide titration assays to be a weaker agonist than p33 because it binds relatively poorly to D\textsuperscript{b}.

The weak agonists W4Y, A4Y, and L6F and antagonist G4Y behaved in these assays with the expected short $t_{1/2}$ and lower avidities. The avidities of weak agonists were, however, not much lower than those of strong agonists. SPR analyses have shown that affinity is not always a strong predictor of biological activity and that the affinities of strong and weak agonists can be quite close (8–11). In contrast, the $t_{1/2}$ of all of the weak agonists was clearly lower than strong agonists. In all assays, the antagonist G4Y had the lowest avidity, ability to inhibit tetramer binding, and $t_{1/2}$ of the APLs. The ability of the weak agonists to compete for tetramer binding was significantly lower than the avidity measurements would suggest (since both are related to affinity), indicating that tetramer avidity may not be the best measure for these comparisons.

Only the natural peptide mDBM behaved completely unexpectedly. It had a relatively high avidity and long half-life that would group it with the strong agonists, although it is a positive selector and a partial agonist with some antagonist properties and a low ability to down-modulate TCR (Refs. 29, 32, and 33 and Fig. 4). The RMA-S MHC stabilization assay showed that mDBM bound D\textsuperscript{b} with a much lower affinity than p33. As with A3V, the biological response may be the result of opposing forces, the strong interaction between TCR and mDBM-D\textsuperscript{b} modulated by the weak interaction between mDBM and D\textsuperscript{b}. In experiments with soluble peptide-D\textsuperscript{b} complexes, the effect of the low-affinity interaction between the mDBM peptide and D\textsuperscript{b} is reduced because the soluble pMHC complexes only stay together when peptide is bound. By normalizing the D\textsuperscript{b} presentation of different peptides using RMA-S cells, we found that mDBM in fact has a relatively strong ability to stimulate T cells (Fig. 6) in accord with its tetramer avidity. In systems where mDBM competes with endogenous peptides that may have a higher affinity for MHC, its response is therefore diminished. This could explain the positive selection and weak agonist characteristics of this peptide and highlights the fact that the efficacy of an APL depends not only on the strength of the TCR-pMHC interaction, but also on that between the peptide and the MHC. This point has perhaps not been given sufficient weight in many previous studies, possibly because most analyses on series of APLs have dealt with single amino acid variants of the antigenic peptide. However, its importance in determining the efficacy of a pMHC ligand has recently been shown in another TCR-pMHC system (13). Also, it has been noted that anomalies in the off-rate: biological response relationship are frequently seen in series of APLs that differ in MHC-interaction residues (8, 10, 11). Finally, tetramer avidity, while good for comparing strong/weak binding of pMHC to TCR, is not as good for comparison of series of ligands where differences in affinity are more subtle. In these cases, differences in $t_{1/2}$ and tetramer binding inhibition may be more useful.

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References


