Recall the reference to page 47320, DOI 10.1074/jbc.M109231200

The interaction between a TcR and class I MHC is not an “all or nothing” event. Altered peptide ligands (APLs) that only differ slightly from the original peptide lead to a continuum of different effects when presented to T cells (2, 3). Based upon the outcome of T cell activation, APLs may be defined as agonists or super agonists, which trigger similar or improved T cell responses compared with the original peptide ligand. APLs may also be null peptides that completely fail to stimulate any response either because of a failure to produce a productive signal upon engagement of the TcR or a failure to interact with the TcR at all. Furthermore, APLs may be antagonists. These APLs inhibit the ability of CTL to kill cells bearing the natural agonist peptide.

The critical physical constants between pMHC and TcR that control the triggering of T cell activation are unknown. Early experiments using live T cells and antigen-presenting cells equated T cell activity to affinity of TcR for pMHC (2). However, a number of surface plasmon resonance experiments with soluble forms of pMHC and TcR indicate that the differences in the activity of agonist, antagonist, and null pMHC complexes are primarily due to variations in the off rates, or half-life, of the interaction (4–7). Finally, some researchers have found that neither affinity nor kinetics correlate with the differences in T cell outcome (8). Although surface plasmon resonance experiments are valuable because they allow us to determine the kinetics and thermodynamics of the interaction between pMHC and TcR, these measurements fail to account for the involvement of co-receptors and accessory molecules on the cell surface that are also critical in T cell and antigen-presenting cell interactions, particularly when the affinity of the TcR to pMHC complex is low (9–12). In addition, other physical interactions may be important for T cell activation including TcR clustering and dimerization, co-receptor recruitment, and costimulation by accessory molecules. Indeed, many structures of TcR in complex with class I MHC have been determined to date (16–22). However, a number of surface plasmon resonance experiments with soluble forms of pMHC and TcR indicate that the differences in the activity of agonist, antagonist, and null pMHC complexes are primarily due to variations in the off rates, or half-life, of the interaction (4–7). 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In an attempt to determine a physical mechanism that controls the signals that result in different T cell activities, the crystal structures of TcR A6 in complex with four different peptide-A2 complexes (one agonist, one weak agonist, and two weak antagonists) have been determined (17). No physical mechanism is immediately apparent; the crystal structures of these complexes are almost identical. There are no significant conformational changes in the domain structure of the TcR that...
Recognition of APL Depends on pMHC Affinity

Changes to the p1049 and p1058 wild type residues are shown in boldface type. $K_{i}$ is the concentration of peptide in $\mu M$ that yields 50% of maximal binding of p1049 to A2 on the surface of T2 cells (Fig. 2A). AHIII12.2 (5:1 E:T ratio) were added to EE2H3 cells transfected with an A2-$\beta_{2}$m fusion protein pulsed with 20 $\mu M$ of the indicated peptide. $^{31}$Cr release was measured 4 h later. CTL is the percentage of specific lysis, where specific lysis is normalized to the percentage of specific lysis of p1049 in each experiment (Fig. 1A). T cell outcome was assessed based on the outcome of the antagonism assay (Fig. 1B). ND, not determined.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>$K_{i}$</th>
<th>CTL</th>
<th>T cell outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>p1049</td>
<td>ALWGFFPVL</td>
<td>0.7</td>
<td>100</td>
<td>Strong agonist</td>
</tr>
<tr>
<td>p1049-F3V</td>
<td>ALWGFFPVL</td>
<td>0.7</td>
<td>0</td>
<td>Null</td>
</tr>
<tr>
<td>p1049-F6V</td>
<td>FLWGFFPVL</td>
<td>5.0</td>
<td>83</td>
<td>Weak agonist</td>
</tr>
<tr>
<td>p1049-A1F</td>
<td>FLWGFFPVL</td>
<td>0.6</td>
<td>71</td>
<td>Strong agonist</td>
</tr>
<tr>
<td>p1049-W3P</td>
<td>ALPGFFPVL</td>
<td>4.5</td>
<td>13</td>
<td>Weak antagonist</td>
</tr>
<tr>
<td>p1049-V8Y</td>
<td>ALWGFFPVL</td>
<td>1.6</td>
<td>94</td>
<td>Strong agonist</td>
</tr>
<tr>
<td>p1049-Y18T</td>
<td>ALWGFPPTL</td>
<td>1.9</td>
<td>118</td>
<td>Strong agonist</td>
</tr>
<tr>
<td>p1049-A1F/V8Y</td>
<td>FLWGFPPTL</td>
<td>0.5</td>
<td>20</td>
<td>Strong antagonist</td>
</tr>
<tr>
<td>p1049-W3L/V8Y</td>
<td>ALLGFPPTL</td>
<td>1.1</td>
<td>25</td>
<td>Weak antagonist</td>
</tr>
<tr>
<td>p1049-W3P/V8Y</td>
<td>ALLGFPPTL</td>
<td>1.9</td>
<td>15</td>
<td>Weak agonist</td>
</tr>
<tr>
<td>p1058</td>
<td>FAPGFFPVL</td>
<td>7.9</td>
<td>29</td>
<td>Strong antagonist</td>
</tr>
<tr>
<td>p1058-P3I</td>
<td>FALGFFPVL</td>
<td>1.5</td>
<td>21</td>
<td>Strong antagonist</td>
</tr>
<tr>
<td>p1058-P3W</td>
<td>FAWGFFPVL</td>
<td>0.8</td>
<td>23</td>
<td>Weak antagonist</td>
</tr>
<tr>
<td>p1058-P3F</td>
<td>FAPGFPVL</td>
<td>2.3</td>
<td>10</td>
<td>Null</td>
</tr>
<tr>
<td>p1058-P3T</td>
<td>FAPGFPPTL</td>
<td>2.9</td>
<td>2</td>
<td>Null</td>
</tr>
<tr>
<td>p1058-A1F/P3W</td>
<td>AAWGFFPVL</td>
<td>1.6</td>
<td>70</td>
<td>Weak agonist</td>
</tr>
<tr>
<td>p1058-P3F/Y8V</td>
<td>FAPGFPVL</td>
<td>3.1</td>
<td>3</td>
<td>Weak agonist</td>
</tr>
<tr>
<td>p1058-P3W/Y8V</td>
<td>FAWGFPVL</td>
<td>1.2</td>
<td>33</td>
<td>Weak antagonist</td>
</tr>
<tr>
<td>MLL</td>
<td>MLLSVPLL</td>
<td>1.8</td>
<td>ND</td>
<td>Irrelevant</td>
</tr>
</tbody>
</table>

are triggered by engagement of an agonist versus antagonist that may explain the differing biological consequences. Although these findings show that T cell outcome is not a direct consequence of universal structural changes, this does not rule out the possibility that small, localized changes to the TcR binding surface could affect the affinity or kinetics of the pMHC-TcR interaction, which in turn may have an effect on the resulting T cell outcome. For example, the structures of antagonist human immunodeficiency virus-1 gag peptides bound to HLA-B$8$ exhibited changes in the peptide, to the MHC as a result of changes in the peptide, and to the antigen-binding cleft (23), suggesting that there were alterations in the molecular structure linked to biological activity. Additional crystal structures are required to probe each possibility.

The murine AHIII12.2 T cell clone recognizes class I molecule H-2D$^b$ (D$^b$) in complex with a synthetic peptide p1058 (sequence FAPGFFPYL) and several variants based on this sequence (24). Interestingly, this murine CD8 T cell clone also recognizes a xenogeneic (human) class I MHC HLA-A2.1 (A2) in complex with peptide p1049 (sequence ALWGFFPVL) (25). Our previous comparison of the x-ray crystal structures of A2-p1049 and D$^b$/p1027 (also recognized by AHIII12.2) demonstrates that the molecular surfaces recognized by this TcR are not similar (26), indicating that molecular mimicry does not play a significant role in the recognition of dissimilar pMHC by this T cell clone. Exactly how the TcR could recognize two very different molecular surfaces is still not known.

While examining the recognition of altered peptide ligands by AHIII12.2, we found that the D$^b$-binding peptide, p1058, also binds to HLA-A2.1. Furthermore, when bound to A2, p1058 is antagonistic to p1049 function on AHIII12.2 T cells. To explore the differential recognition of p1049 and p1058 when bound to A2, we synthesized a panel of chimeric peptides derived from p1058 that progress to p1049 and vice versa. Substitutions in p1049 or p1058 result in agonist, antagonist, and null peptides. To probe molecular recognition of xenogenic class I MHC, we determined the structures of an antagonist (p1058), a weak agonist (p1049-F6V), and a null peptide (p1049-F5V) bound to A2. In results similar to those obtained by the Ding et al. studies (17), and unlike the Reid et al. study (23), we see no large structural changes that suggest reasons for the differences in biological activity. However, the structures do provide a rationale for differences in activities observed. Furthermore, using multimeric pMHC complexes, we find that the differences in biological activity may be explained by differences in affinity of the pMHC antigen for the TcR on the surface of AHIII12.2 T cells.

MATERIALS AND METHODS

Peptides—The Peptide Synthesis Facility at the National Cancer Institute (National Institutes of Health) synthesized all peptides used in this study. The peptides were purified by reversed-phase HPLC (>95%), and their identity was confirmed by laser desorption time of flight mass spectrometry. The purified peptides were dissolved in 100% Me$_2$SO at 20 mg ml$^{-1}$ by weight. The sequence of each peptide is given in Table I.

CTLAssays—Lytic activity of CTL toward the altered peptides was performed by a standard 4-h $^{31}$Cr release assay as described previously (24). Briefly, between 1.5 $\times$ 10$^5$ and 2.5 $\times$ 10$^5$ AHIII12.2 T cells were incubated with 5000 peptide-pulsed $^{31}$Cr-labeled target cells as described in the legend to Fig. 1 and Table I. Since p1049 is a human self-antigen, human cell lines could not be used to assay CTL activity of variant peptides. Another benefit of using murine cells is that the xenogenicity eliminates many of the differences among auxiliary molecules that might also influence the quality of the TcR-pMHC interaction. Murine EE2H3 cells, which do not normally express any class I MHC (27), were transfected with an A2 heavy chain covalently linked to human $\beta_{2}$m and used as target cells (28).

Antagonist activity was assayed as described previously (3). Briefly, this entailed incubating target cells (either EE2H3 or CHO-A2) with a suboptimal concentration of p1049 (50 nM) for 2 h, to generate 20–40% of the maximum specific lysis of AHIII12.2. Cells were washed and plated to microwells containing a 10 $\mu M$ concentration of the test peptide or no peptide. After a further 1-h incubation, CTL activity was measured as described above. The percentage of inhibition was calculated as in Ref. 29.

Peptide-dependent Class I MHC Stabilization Assay—Cell surface stabilization of A2 in the presence of variant peptides was performed as previously described (30). Briefly, 2.5 $\times$ 10$^5$ T-2 cells (ATCC number CRL-1992) were incubated overnight in AIM V serum-free medium (Life Technologies, Inc.) at 37 $^\circ$C in 5% CO$_2$ in the presence of 100 nM human $\beta_{2}$m and peptide at concentrations from 10 to 0.1 $\mu M$. Cells were then stained with the monoclonal antibody BB7.2, specific for HLA-A2, followed by fluorescein isothiocyanate-labeled goat anti-mouse IgG (Southern Biotechnology Associates). Cells were analyzed using flow cytometry (FCScan; Becton Dickinson), and the mean channel fluorescence was determined using the CYCLOPS software package (Cytof- mation; Fort Collins, CO). All data were normalized to the percentage of mean channel fluorescence for p1049 bound to A2 at 10 $\mu M$.

Cell Surface Class I MHC Half-life Assay—The determination of cell
Table II
Summary of crystallographic and refinement statistics

<table>
<thead>
<tr>
<th>A2-p1059-F6V</th>
<th>A2-p1059-F5V</th>
<th>A2-p1058</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution (Å)</td>
<td>50–1.85</td>
<td>50–2.8</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>4.0 (17.8)</td>
<td>14.8 (37.9)</td>
</tr>
<tr>
<td>(I/s)</td>
<td>9.3 (2.7)</td>
<td>7.0 (9.0)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>97.2 (95.5)</td>
<td>96.3 (96.7)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>2.0</td>
<td>3.9</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>23.6</td>
<td>22.9</td>
</tr>
<tr>
<td>Rfree (%)</td>
<td>25.8</td>
<td>28.5</td>
</tr>
<tr>
<td>Error (Å)</td>
<td>0.12</td>
<td>0.27</td>
</tr>
<tr>
<td>RMSD (bonds) (Å)</td>
<td>0.91</td>
<td>0.76</td>
</tr>
<tr>
<td>RMSD (angles) (Å)</td>
<td>0.91</td>
<td>0.76</td>
</tr>
<tr>
<td>No. of wafers</td>
<td>489</td>
<td>39</td>
</tr>
</tbody>
</table>

\( R_{merge} = \frac{\sum_{hkl} \sum_{i} |I_{i} - \langle I \rangle|}{\sum_{hkl} \sum_{i} I_{i} \langle I \rangle} \), where \( I \) is the observed intensity.

\( R_{merge} \) was calculated for a randomly chosen 5\% of reflections.

Redundancy was calculated as the average linear space correlation coefficient on all atoms per residue based on DM-modified electron density maps.

Average redundancy is the numerical average of all observations divided by unique observations. RMSD is root mean square deviation.

Number in parentheses refers to the highest resolution shell (2.24–2.20Å (p1058), 1.88–1.85Å (p1049-F6V), and 2.85–2.75Å (p1049-F5V)).


db-mHC complexes—Large quantities of heavy chain residues 1–274 and human \( \beta_{2m} \) were produced as inclusion bodies in Escherichia coli (26), purified, and folded in vitro as previously described (31). The folded db-mHC complexes were concentrated using an ultrafiltration cell (Amicon) and purified by HPLC gel filtration chromatography (Phenomenex, Biosep-SEC-S2000). All db-mHC complexes were crystallized using the hanging drop vapor diffusion method. The reservoir for A2-p1059 contained 14\% polyethylene glycol 6000 in 25 mM MES buffer, pH 6.5. The reservoir solution for A2-p1049-F6V and p1049-F5V is similar to that for A2-p1058, with the exception of 16\% polyethylene glycol 6000 rather than 14\%. The hanging drop for every complex was a 1:1 mixture of the reservoir solution and the protein solution, which contained 10 mg/ml protein in 25 mM MES buffer, pH 6.5. Microseeding was necessary for all complexes to obtain crystals suitable for crystallographic studies.

Structural Determination and Refinement—Crystals of db-mHC complexes were stored in 25 mM MES, pH 6.5, containing 20\% polyethylene glycol 6000. These crystals were transferred directly into the storage buffer plus 25\% glycerol and were immediately placed in the cryostream (generated by the Oxford Cryo System, 100K). The diffraction data for A2-p1058 and A2-p1049-F5V were collected at the National Synchrotron Light Source at the Brookhaven National Laboratory on station X12-B, and the diffraction data for p1049-F5V were collected on a Rigaku R-Axis IIC using Cu Ka radiation. All diffraction data were processed using the programs Denzo and Scalepack. Data statistics are shown in Table II.

The structures of all complexes were determined using the Molecular Replacement method with the CCP4 program suite. The complex of A2 and a peptide from human immunodeficiency virus reverse transcriptase (32) was used as a model for all three structural solutions. The orientation and position of these models were determined using the program AMoRe in CCP4. Solvent flattening and 2-fold averaging using the program DM in the CCP4 suite were applied to improve the quality of the density. Manual model building into the electron density was performed with the program O (33).

Computational refinement was performed using the program CNS (34). Rigid body refinement was carried out using three domains: \( \alpha_{C}, \alpha_{B}, \) and \( \beta_{2m} \). Simulated annealing was used at early stages of the refinement. Energy minimization was carried out using the maximum likelihood target function. Bulk solvent and anisotropic B corrections were applied through the refinement process. Cycles of manual model rebuilding and refinement were carried out until \( R_{merge} \) and \( R \) factors were converged. Water molecules were then added with ARP (35). Individual \( B \) factor refinement was performed as the final step of refinement. Refinement was continued with added water until \( R_{merge} \) converged. The final refinement statistics are shown in Table II.

Biotinylation of pMHC Complexes and Octamer Formation—Biacore mHC protein was prepared as described above, except the MHC heavy chain contained a BiA recognition sequence at the C terminus that allowed for site-specific biotinylation. Purified pMHC complexes were biotinylated using the methods provided by the manufacturer (Avidity, Denver, CO). Excess biotin was removed with three subsequent buffer exchanges in a Centriprep filtration device (Amicon) with 10-kDa membrane. Incubation of biotin-labeled protein with streptavidin (Sigma), followed by SDS-polyacrylamide gel electrophoresis without boiling or reducing agent, was used to assess the extent of biotinylation. Properly biotinylated pMHC complexes supershift with streptavidin added.

To make pMHC tetramer complexes, avidin-PE (Leinco, St. Louis, MO) was added to biotinylated pMHC monomers. After a 15-min incubation on ice, monoclonal anti-PE antibody (Sigma) was added to the tetramers to form octamers.

Affinity and Half-life Assays—For binding assays, the octamer complexes were added at the indicated concentrations to 2 \times 10^6 AHI112.2 T cells. After a 45-min incubation at 4°C, cells were washed three times and analyzed for median fluorescence by flow cytometry (FACScan; Becton Dickinson).

For half-life assays, 8 \times 10^6 AHI112.2 T cells were stained with 350 nM of the indicated octamers for 45 min at 4°C. Cells were washed three times and then resuspended in a solution containing the monoclonal antibody BB7.2 (purified from hybridoma supernatant) at 50 \mu g/ml. At the indicated time points, aliquots of the cells were added to wash buffer containing 1% formalin and analyzed for median fluorescence by flow cytometry (FACScan; Becton Dickinson).

As a negative control in both experiments, octamers composed ofHLA-A2.1 complexed to an irrelevant peptide (MLLSVPLL) were used. The median fluorescence of the A2-MLL octamers at each concentration or time point was subtracted in each experiment.

RESULTS

Altered Peptide Ligands Based on p1049 or p1058 Cause a Variety of T Cell Outcomes—During our studies of AHI112.2 recognition of structurally disparate ligands, it was discovered that p1058, recognized as an agonist by AHI112.2 when bound to D\( ^{\alpha} \), also binds to the human class I MHC A2 and is recognized weakly (Fig. 1A). In order to study reactivity of AHI112.2 T cells to the two different ligands when bound to A2, a chimeric series of peptides were constructed that progressively changed p1049 into p1058 and conversely p1058 into p1049 as shown in Table I. These peptides showed various abilities to be recognized by AHI112.2 (Fig. 1A) in a standard 4-h \( ^{51} \)Cr release assay. Since the central two residues are the same between the two peptides, substitutions were made at positions P1, P3, and P8 in the chimeric series. Substitutions at the anchor positions (36) were avoided to minimize large differences in peptide binding affinity to A2.

Considering only P1, P3, and P8 substitutions (Fig. 1A and Table I), the greatest changes in recognition observed for p1049-based variants are found at the secondary anchor P3 (Tyr to Pro) that caused a nearly 10-fold drop in CTL activity (p1049-W3P). This result is perhaps not surprising considering the differences the proline linkage could be expected to make to the peptide main chain. In addition, the proline could not occupy the large hydrophobic D pocket (37) in any fashion similar to tryptophan. As a consequence, this substitution would be expected to result in large conformational changes in the peptide. However, this interpretation will be revisited later.
under Discussion. Alterations in the P1 residue cause decreased AHIII12.2 recognition (p1049-A1F), but to a lesser extent than those seen at P3. Interestingly, alterations at P8 also seem to play a significant role, since p1049-V8T shows enhanced recognition relative to p1049. Combinations of substitutions generate unexpected changes. Since p1049-V8Y had a subtle to nonexistent affect on recognition and p1049-A1F had a modest decrease in reactivity, a small to modest decrease in reactivity when combining the substitutions (p1049-A1F/V8Y) was expected. However, the doubly substituted peptide has 5-fold reduced recognition compared with p1049, indicating that the combined effects on T cell activation are not simply additive.

Substitutions in p1058 generate conflicting data regarding AHIII12.2 recognition relative to the p1049 substitutions. In p1049, the Trp to Pro substitution at P3 results in a 10-fold reduction in recognition. The converse substitution, p1058-P3W, does not greatly improve recognition by AHIII12.2, nor do similar substitutions that could alter subtle side chain interactions, particularly from the Phe side chain at P5 (p1058-P3L, p1058-P3F). The substitution p1049-V8T improves recognition of p1049 but completely abolishes recognition of p1058 (p1058-Y8T). Substitution of P1 to Ala in p1058 results in a substantial increase in recognition (compare p1058-P3W with p1058-F1A/P3W), suggesting that the P1 residue is critical for recognition by the AHIII12.2 TcR, but the complementary substitution (p1049-A1F) causes only modest effects. The differences in peptides p1058-F1A/P3W (AAGFPPPFL) and p1049-V8Y (ALWGFPFYL) illustrate that the P2 anchor substitution Leu to Ala does affect recognition (70 and 94%, respectively) as expected. Combined, these data illustrate that recognition of particular peptide residues is different between p1058 and p1049 when bound by the same protein (A2) and recognized by the same TCR (AHIII12.2).

Chimeric Peptides Generate Phenotypic Changes to AHIII12.2—Various altered peptide ligands in the literature have been shown to cause changes in T cell outcomes such as partial agonism and antagonism (2, 3, 38). The chimeric series
of peptides in Table I were tested in an antagonism assay (Fig. 1B; summarized in Table I). The antagonist activity observed was not due to competition for binding to A2 (or similarly to the TcR) because the assay uses minimal agonist peptide. A2-expressing fibroblasts were pulsed with suboptimal concentrations of p1049 peptide, washed, and incubated with increasing concentrations of test peptide (i.e. p1058 or others in Table I). Again surprisingly, p1058 stands out because it is antagonistic to p1049 recognition. Increasing lysis as a function of the concentration of added test peptide defines an agonist. Furthermore, a strong agonist is a peptide that generates a greater than 50% enhancement in percentage of specific lysis relative to base line; a weak agonist generates less than a 50% increase in percentage of specific lysis. Decreasing lysis defines an antagonist (again greater than 50% decrease is a strong antagonist and less is a weak antagonist). Peptides that generated less than a 10% change in enhancement or inhibition are classified as null peptides. As can be seen in Table I, a variety of T cell outcomes are observed with the chimeric peptides. By these criteria, of the 19 peptides tested, four are strong agonists, with p1049 and p1049-VST being the strongest. Three of the peptides are strong antagonists, with p1058, p1049-A1F/V8Y, and p1058-P3L all equally strong. The weak agonists and antagonists have a distribution of enhancement or inhibition. As may have been expected, these outcomes correlated closely with the results of the standard CTL assay, since peptides that resulted in good lysis in the standard CTL assay are strong agonists, while those peptides that had minimal lysis in the standard CTL assay are antagonist or null peptides.

**Differences in Biological Activity Do Not Correlate with Variant Peptide Binding Constants**—One potential explanation for the observed differences in T cell reactivity is differences in the binding affinity of the peptides to the class I MHC, A2. Simply put, the T cell clone might not recognize peptides that bind poorly to A2. Relative peptide binding was examined by incubating T2 cells defective in antigen processing with increasing concentrations of exogenous peptide. T2 cells have large amounts of peptide receptive A2 on the cell surface, and binding of exogenously added peptide may be measured by the amount of A2 that remains on the cell surface using flow cytometry. This binding has been shown to be proportional to affinity binding constants (39). As can be seen in Fig. 2A, the binding of p1058 to A2 on the surface of T2 cells is 5-fold worse than p1049, suggesting that antagonism might be generated under conditions of low peptide concentration on the surface of the antigen-presenting cell. However, examination of peptide binding for the series of chimeric peptides indicates no correlation between the affinity of peptide binding to A2 and lytic activity (Fig. 2B and Table I). For example, p1049-A1F/V8Y binds as well as or better than all agonists but is recognized as poorly as p1058 (interestingly, it is also a strong antagonist). Although the affinity of some of the variant peptides for A2 is much weaker than others in this study, all are well within the range of good binding peptides (40). In addition, the rate of dissociation of the peptides once bound to A2 does not correlate with activity and T cell outcome of the peptides (Fig. 2C). Dissociation of the peptide-MHC complex can be measured on the surface of T2 cells after peptide pulsing in the presence of brefeldin A. Examination of the binding of other chimeric peptides showed that there is no correlation observed for the affinity or dissociation kinetics of peptide binding to A2 for each of the agonist, antagonist, and null peptides (Fig. 2 and Table I). Specifically, p1049-A1F/V8Y is an antagonist, as is p1058, but p1049-A1F/V8Y binds better than p1049 and has a longer half-life (t is 20 h for p1049 as compared with 32.7 h for p1049-A1F/V8Y). Thus, the agonist, antagonist, and null T cell outcomes are observed with the chimeric peptides. By these criteria, of the 19 peptides tested, four are strong agonists, with p1049 and p1049-VST being the strongest. Three of the peptides are strong antagonists, with p1058, p1049-A1F/V8Y, and p1058-P3L all equally strong. The weak agonists and antagonists have a distribution of enhancement or inhibition. As may have been expected, these outcomes correlated closely with the results of the standard CTL assay, since peptides that resulted in good lysis in the standard CTL assay are strong agonists, while those peptides that had minimal lysis in the standard CTL assay are antagonist or null peptides.

**Recruitment of APL Depends on pMHC Affinity**

![Fig. 2. T cell outcome does not correlate with peptide binding to HLA-A2.1.](http://www.jbc.org/)

A: binding to cell surface A2 on T2 cells shows no correlation with biological activity. T2 cells incubated with the indicated concentration of peptide overnight were stained with BB7.2 and visualized by flow cytometry. p1049 (■), p1049-A1F (∆), p1049-VST (●), p1049-W3P (○), p1058 (□), p1058-Y8T (▲), p1058-P3L (▲′), p1058-W3F (▲′′), p1049-W3P (▲′′′), p1049-F6V (▲′′′′), p1049-F5V (▲′′′′′), p1049-V8T (▲′′′′′′), p1049-A1F/V8Y (▲′′′′′′′), p1058-P3W (▲′′′′′′′′), and p1058-P3W (▲′′′′′′′′′) are shown. B, a scattergram of CTL activity versus relative binding capacity (K_r) shows the lack of correlation (R value 0.0961). C, cell surface half-life assay shows no correlation with T cell outcome. Brefeldin A-treated T2 cells pulsed with peptide were stained with BB7.2 at the indicated times. The amount of A2 remaining on the cell surface over time was visualized by flow cytometry. p1049 (■), t = 20 h; p1049-F5V (▲), t = >40.0 h; p1049-F6V (○), t = >40.0 h; p1049-A1F/V8Y (●), t = 32.7 h; p1058 (□), t = 1.3 h.
outcomes observed do not appear to be due to differences in peptide binding affinity or dissociation kinetics to A2.

Structures of Antagonist, Agonist, and Null Complexes—In order to examine whether there are physical differences that could account for differences in biological activity, the crystallographic structures of three peptides bound to A2 were determined. An antagonist (p1058), a weak agonist (p1049-F6V), and a null (p1049-F5V) peptide were chosen.

The heavy chain structure of the antagonist A2-p1058 (FAPGFFPYL) is nearly identical to the full agonist A2-p1049 (ALWGFFPVL). The root mean square deviation is 0.46 Å. The main chain conformation of peptide p1058 is also nearly identical to p1049, although the substitutions at the P1-P3 end are dramatic (ALW in p1049 and FAP in p1058) (Fig. 3A). This is surprising, because smaller substitutions have caused much larger changes in the structures of pMHC (41). In particular, it was expected that the substitution of Pro for Trp at P3 would cause main chain differences because of the proline linkage. However, the dihedral angles required to generate the kink seen in the main chain between residues P3 and P4 (in nearly all peptides bound to A2) is allowed for proline (42). In addition, the lack of the side chain in the D pocket (37) is compensated in an unexpected fashion. The most notable difference in the peptide-binding cleft of A2 is a slight adjustment of the TyrA99 side chain. The phenol ring of heavy chain residue TyrA99 in the A2-p1058 complex swings roughly 15° to make room for ProP3 in the p1058 peptide. In addition, the Phe at P5 of p1058 rotates down into the D pocket, filling the cavity created by the missing Trp and shown in Fig. 3A by the overlap of the Trp at P3 of p1058 and Phe at P5 in p1049.

The heavy chains in the A2-p1049-F5V and A2-p1049-F6V structures are essentially identical to A2-p1049 (26); the root mean square deviation from A2-p1049 is 0.24 and 0.32 Å, respectively. Again surprisingly, the main chains of the peptides are also nearly identical to p1049 (Fig. 3, B and C). In retrospect, this perhaps is not surprising for p1049, p1049-F5V, and p1049-F6V, since the differences between these peptides are both small changes to solvent-exposed residues (both change from Phe to Val (p1049: ALWGFFPVL)). As a result, the only structural difference among A2-p1049, A2-p1049-F5V, and A2-p1049-F6V is the presence or absence of the phenyl ring at P5 or P6. There are very small differences in the peptide-binding pocket between F5V and F6V that are the result of the presence of the different side chains in the peptide. The imidazole ring of HisA74 residue on the α1 helix undergoes a 45° rotation around the Cγ and Cβ axis in the A2-p1049-F5V structure and a nearly 90° rotation in the A2-p1049-F6V structure when compared with A2-p1049. However, these changes do not generate significant differences in the molecular surfaces (data not shown). Thus, while there are differences in the structures that may help interpret the biology, they are not significant differences. These changes cause very conservative differences in structure.

An interesting comparison may be made between the peptide main chain of p1058 (FLPGFFPVL) bound to A2 and p1027 (FLPGVFPYM) bound to D4 (26). When bound to D4, p1027 and p1058 are both agonists of similar strength, suggesting that they assume similar three-dimensional structures. However, when the p1058 peptide is bound to A2, it takes a very different main chain orientation (Fig. 3D). This suggests that the sequence of the class I MHC plays a large role in the conformation of the peptide.

TcR Binding Affinity and Half-life as Assessed by Multimeric pMHC Association—There are a variety of models available for generation of T cell outcome based on affinity or kinetics of association between pMHC and TcR (4–8, 13–15). We hypothesized that the binding affinity of AHIII12.2 for agonist pMHC complexes would be higher than that observed for null and antagonist complexes and may correlate with activity. In order to test this, pMHC tetramers were constructed, and binding
was measured to AHIII12.2 T cells using flow cytometry. These types of assays have been used to determine relative affinities (43). Unfortunately, only tetramers composed of A2-p1049 and A2-p1049-V8T bound to any significant extent (data not shown). Since it has been shown that affinity for the TCR increases with the valency of pMHC (13, 14), an octameric form of the pMHC complexes was produced using a monoclonal antibody to phycoerythrin. All octamers composed of agonists, except p1049-F6V, bound to the AHIII12.2 T cell, but binding was not observed for any of the null or antagonist pMHC complexes (Fig. 4A). Scatchard analysis was performed for the octamers that bound (Fig. 4B), and a K_d for octamer binding was calculated from the inverse of the slope of the least squares fit to the data. Curves are the average of at least three separate experiments, and the associated error bars are smaller than the symbol for the data. Octamers dissociation was measured by the addition of saturating concentrations of blocking antibody as described previously (44). t_1/2 was calculated as ln 2 divided by the negative slope of the least squares fit to the data. Octameric off rates do not correlate with T cell lysis or T cell outcome. Curves are the average of three separate experiments.

Recognition of APL Depends on pMHC Affinity

Fig. 4. Differences in T cell outcome and CTL reactivity correlate well with octamer binding to AHIII12.2 T cells. A, binding of octameric complexes to AHIII12.2 cell surface. AHIII12.2 T cells were incubated with a 700 nM concentration of the indicated octameric complex, and median fluorescence was measured by flow cytometry. Median channel fluorescence of each complex was normalized to the median fluorescence of p1049 at saturating concentrations. Data shown for p1058 are representative of all octameric complexes made from peptides classified as antagonist or null in Table I. B, Scatchard plots of pMHC agonist octamers binding to AHII 12.2 T cells. Median fluorescence values were normalized to p1049 median fluorescence at saturation after background subtraction within each experiment. Concentrations of octamers were normalized to one experiment to account for variations in PE-conjugated ultra-avidin activity in different batches from the manufacturer. The K_d was calculated from the inverse of the slope of the least squares fit to the data. Curves are the average of at least three separate experiments, and the associated error bars are smaller than the symbol for the data. C, a scattergram of lytic activity (CTL in Table I) versus octamer binding (K_d) shows good correlation (R value = 0.849) between lysis of agonists and affinity between pMHC and TcR. D, octamer dissociation rates are fast (t = 60–90 s) and do not vary greatly. Octameric dissociation was measured by the addition of saturating concentrations of blocking antibody as described previously (44). t_1/2 was calculated as ln 2 divided by the negative slope of the least squares fit to the data. Octameric off rates do not correlate with T cell lysis or T cell outcome. Curves are the average of three separate experiments.
linked to differences in off-rate. However, it must be stated that it is unclear how the rates of dissociation of an octamer relate to the rates of dissociation of a monomer.

**DISCUSSION**

The recognition of antigen by T cells is a complex reaction involving adhesion and co-stimulatory molecules, co-receptors, and the interaction between TcR and pMHC. Discrimination of recognition is entirely determined by the interaction between pMHC and TcR. In our studies probing reactivity of the xenoreactive T cell clone AHIIII12.2, it was found that the Dβ-binding peptide also binds to the human class I MHC A2. In order to better understand recognition of p1058 and p1049 by the AHIIII12.2 T cell in the context of A2, a series of chimeric peptides was synthesized. These chimeric peptides, when bound to A2, are recognized variably. A result not anticipated was that similar substitutions generated different affects on recognition depending on the context of the peptide in which the substitution was made. For example, replacing the P8 position with threonine resulted in contrary effects depending on whether it was substituted in p1049 or p1058. In p1049, the V8T substitution increases recognition (lysis) by AHIIII12.2 T cells. In p1058, the Y8T substitution results in significantly decreased recognition. Therefore, the conclusion must be that although p1049 and p1058 are both presented to the TcR by the same MHC (A2), the way the AHIIII12.2 TcR interacts with the MHC is different in the two cases. The results are similar to the recognition of two very different complexes of Dβ-p1058 and A2-p1049 by AHIIII12.2 that we have described previously (26), only apparently with a smaller number of sequence differences.

The structures of representative complexes, p1058 (antagonist), p1049-F5V (null), and p1049-F6V (agonist) were determined. Although the structures are remarkably similar, they provide data that explain the different biology. For example, the substitution of p1049-W3P results in a 10-fold reduction in lysis by AHIIII12.2 T cells. A reasonable hypothesis is that proline dramatically alters the main chain conformation. However, the structure of p1058 bound to A2 shows that proline is capable of generating the main chain path taken by p1049 with a tryptophan at P3. The probable explanation for the differences in recognition is that the rotation of the P5 Phe fills the hydrophobic cavity (pocket D) (37) normally filled by the tryptophan. The P5 residue is directly in the center of the peptide-binding groove. Based on the crystal structures of TcR and pMHC, P5 is the residue most likely to make contact with the CDR3 loops of the TcR variable domains. The rotamer change that moves that Phe away from the TcR contact site would create a cavity that would reduce binding affinity. This idea is supported by two observations. The first is that p1049-F5V generates no reactivity, which confirms that the size of the Phe side chain is important to TcR recognition. The second observation is that the substitution of Pro at P3 in p1058 (p1058-P3W) shows a slight increase in recognition as opposed to the converse substitution in p1049 (p1049-W3P), which suggests that filling the cavity (by forcing the P5 Phe toward the TcR contact surface) contributes positively.

The presence or absence of a bulky side chain at the P5 position does not explain all of the biological data. The p1058 peptide is an antagonist. The p1058-P3W peptide is also an antagonist (although weaker). Therefore, there must be other residues that are involved in binding that contribute to differences in binding between pMHC and TcR and alter T cell outcome. The most promising candidate is the P8 residue. It is the residue that has the greatest difference in reactivity between the two peptides. It is also the position that causes the greatest shift in outcome in the chimeric series. The peptide p1049-A1F is a strong agonist. The peptide p1049-A1F/V8Y is a strong antagonist. However, the converse substitution, p1058-Y8T, is a null peptide. Therefore, while assignments of relative importance may be made, they are not perfectly assigned to one peptide position versus another. Most likely, the secondary affects on the positions of the nearby amino acids caused by the substitution confound a simple positional analysis.

The structure of p1058 bound to A2 also provides interesting information about class I MHC's ability to alter conformation to accommodate different peptides. The surprise was that the structure does not have to alter conformation dramatically. The antagonist peptide (A2-p1058) main chain path is almost identical to that of the agonist peptide (A2-p1049). This structural similarity is probably a required feature for T cell recognition of these two ligands, but in all of the structures determined to date, there has not been such a similar main chain path with such different sequences. Diversity in T cell outcome as a result of the two different peptides is not due to large structural changes and must then be due to differences in binding affinity or dissociation kinetics between pMHC and the TcR.

Using octamers of soluble peptide antigens, a strong correlation is found between T cell activity, T cell outcome, and peptide/MHC affinity for the T cell receptor on the surface of live T cells. The best CTL activity was observed toward pMHC with the highest affinity. There is range in which small changes in affinity have large changes in T cell activity. Outside of this range, the ability to be recognized is present (albeit not well), and large differences in affinity have little affect on the recognition. It is unclear how the binding constants of the octamers relate to true binding constants of monomers. Other experiments such as those with surface plasmon resonance and soluble TcR are required.

The affinity of the octamers correlated with agonist activity. Null and antagonist pMHC did not bind to the T cell even in an octameric form. Although $K_d$ values of the antagonist and null pMHC complexes are below the limit of detection using this assay, the fact that as a group all antagonist and null complexes do not bind the T cells as octameric complexes and have reactivity less than 40% seems also to suggest that there is an optimal range of $K_d$ values and reactivity for each T cell outcome. Interestingly, it is known that the tetramers of very weak agonists do bind to the T cells, although this binding is below detectable limits by flow cytometry. AHIIII12.2 T cells that are incubated with certain tetramers that do not bind using flow cytometry proliferate as well as they do when presented with A2-p1049 tetramers.

The finding that agonist MHC-peptide complexes with higher CTL activity than antagonist and null complexes also have higher affinity for T cells clashes with current notions of the mechanism of altered peptide ligands in T cell activation. Using soluble recombinant TcR and MHC proteins, several groups have measured affinity and half-lives of agonist and antagonist pMHC complexes for TcR and have come to the conclusion that the off rates of the reaction, and thus the half-lives, are responsible for differences in T cell outcome (4–6, 44). Here considerable differences in affinity (and thus the on rates of the interaction) are seen depending on the strength of the agonist, while the off rates do not seem to vary. Nevertheless, we cannot disregard that we were only able to compare ligands that bound (agonists and weak agonists). Thus, although our findings negate models of T cell activation where the level of T cell activity is dependent upon half-life alone (4–6), they are wholly compatible with the results of Kalergis et al. (44), where an optimal half-life exists for TcR.

[^2]: J. Buslepp, unpublished data.
signaling. However, it is clear that the half-life alone is not the only discriminating factor involved in T cell activity.

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T Cell Activity Correlates with Oligomeric Peptide-Major Histocompatibility Complex Binding on T Cell Surface
Jennifer Buslepp, Rui Zhao, Debora Donnini, Douglas Loftus, Mohamed Saad, Ettore Appella and Edward J. Collins

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