Tax and M1 Peptide/HLA-A2-Specific Fabs and T Cell Receptors Recognize Nonidentical Structural Features on Peptide/HLA-A2 Complexes

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Tax and M1 Peptide/HLA-A2-Specific Fabs and T Cell Receptors Recognize Nonidentical Structural Features on Peptide/HLA-A2 Complexes

William E. Biddison,*2 Richard V. Turner,* Susan J. Gagnon,* Avital Lev,† Cyril J. Cohen,† and Yoram Reiter†

Both TCRs and Ab molecules are capable of MHC-restricted recognition of peptide/MHC complexes. However, such MHC restriction is the predominant mode of recognition by T cells, but is extremely rare for B cells. The present study asks whether the dichotomy in Ag recognition modes of T and B cells could be due to fundamental differences in the methods by which TCRs and Abs recognize peptide/MHC complexes. We have compared MHC and peptide recognition by panels of CTL lines specific for the Tax and M1 peptides presented by HLA-A2 plus Tax and M1 peptide/HLA-A2-specific human Fabs that were selected from a naïve phage display library. Collectively, the results indicate both striking similarities and important differences between Fab and TCR recognition of MHC and peptide components of the Tax and M1/HLA-A2 complexes. These findings suggest that these two classes of immunoreceptors have solved the problem of specific recognition of peptide/MHC complexes by nonidentical mechanisms. This conclusion is important in part because it indicates that Ab engineering approaches could produce second-generation Ab molecules that more closely mimic TCR fine specificity. Such efforts may produce more efficacious diagnostic and therapeutic agents. The Journal of Immunology, 2003, 171: 3064–3074.

The ability of the adaptive immune response to discriminate between self and nonself components, such as foreign pathogens, is determined by the specificity of two classes of immunoreceptors: Ab molecules and TCRs (1, 2). These immunoreceptors have two fundamentally different functions; Ab molecules can recognize both cell surface Ags as well as soluble, free Ags with relatively high affinity, whereas TCRs bind with much lower affinity to cell surface complexes of processed foreign Ag bound by MHC molecules (1, 2). Although functionally distinct, the structures of the immunoreceptors are very similar. Both are heterodimers, with each chain having a variable region that is generated by somatic recombination of multiple germline segments to produce a vast repertoire of different Ag binding sites (1, 2). αβ TCR recognition is almost exclusively restricted to the recognition of peptides presented by MHC molecules, whereas Ab molecules appear to be very limited in their ability to recognize foreign peptide/self MHC complexes. Although various immunization protocols have been shown to elicit Abs that display TCR-like specificity for a particular peptide/MHC complex (3–12), these TCR-like Abs are extremely rare. The present study asks whether the dichotomy in Ag recognition modes of T and B cells could be due to fundamental differences in the mechanisms by which TCRs and Abs recognize peptide/MHC complexes. This question is of more than academic interest because of the potential immunodiagnostic and immunotherapeutic applications of MHC-restricted Abs (9, 11).

Structural studies of αβ TCRs bound to complexes of peptide/MHC molecules have shown that peptide-specific TCRs make multiple contacts with the bound peptide and the α1 and α2 helices of class I MHC molecules (13–23). Approximately two-thirds of the total surface area of class I MHC/peptide complexes contacted by TCRs is contributed by the MHC molecule (24). We have previously shown that the human A6 TCR recognizes the Tax11–19 peptide presented by HLA-A2 (25), and that this TCR binds to the HLA-A2 molecule via contacts on six amino acids on the α1 helix and nine amino acids on the α2 helix (14). To assess the relative contribution of each of these 15 MHC contacts for A6 TCR recognition, alanine scanning mutagenesis was performed on the HLA-A2 molecule at each of these contact residues. Mutant studies showed that for A6 TCR recognition, only three amino acids (R65, K66, and A69) that are clustered on the α1 helix provided the critical interactions for the A6 TCR (26). When these same mutants at positions 65, 66, and 69 were assessed for their effects on recognition by a panel of 201 Tax/HLA-A2-specific CTL lines, the cytotoxicity of each line was negatively affected by at least one of these three amino acid substitutions (26). These results indicated that for all Tax/HLA-A2 TCRs examined, the area around amino acids R65, K66, and A69 provided a critical focus for MHC interaction. These findings were then extended to TCRs that recognized four other peptides presented by HLA-A2, including influenza virus M158–66 (27). Collectively, these results suggested that most HLA-A2-restricted TCRs recognize surfaces on the HLA-A2/peptide complex that are dependent upon the side chains of K66 and Q155 in the central portion of the peptide binding groove (27).

Crystallographic studies of complexes of TCRs and peptide/MHC complexes have also shown that the TCR makes multiple contacts with the bound peptide, and that those side chains of the peptide that project from the central portion of the peptide binding groove make contact with TCR CDR3 loops that form a central
phosphorylation pocket of the TCR. For the Tax peptide, functional studies of altered peptide ligands (APLs)\(^3\) (17, 28–30) and crystallographic studies of TCR/APL/MHC complexes (17) have identified some of the mechanisms by which these peptides produce partial agonist and antagonist effects, including changes in TCR affinity and in signaling pathways. APLs that differentially affect TCR recognition of the influenza M1 peptide have also been described (31, 32). The crystallographic structure of the M1/HLA-A2 complex (33) shows that two of these amino acid substitutions (F5A and F7A) are in the central portion of the M1 peptide and are accessible to the TCR.

Recently a new class of human recombinant Fab Abs has been identified that recognizes tumor-associated and viral-derived HLA-A2-peptide complexes with the same specificity as TCRs (34–37). These Fab Abs were isolated from a large naive library of phage Fabs (38) selected on soluble complexes of HLA-A2 bound to tumor- or viral-associated Ags. These Fabs bound specifically to peptide/HLA-A2 complexes, but not to free peptide, and the binding affinity of these Fabs to the soluble complexes was in the nanomolar range. Previous studies showed that these Fabs are valuable tools for detecting and quantifying specific peptide/HLA-A2 complexes on the surface of tumor cells (34–36) and virally infected cells (37) and determining whether structural alterations in the HLA-A2 molecule could produce conformational changes in the peptide/MHC complex (39). These studies suggest that these Fabs also will provide information that will be relevant to the generation of more effective diagnostic and therapeutic agents.

Among these TCR-like Abs is a panel of Fabs that specifically recognize Tax/HLA-A2 complexes (37). In the present study the same approach as that used to select the Tax/HLA-A2-specific Fabs has been used to select a panel of four different Fabs from this phage display library that exhibit specificity for the M1/HLA-A2 complex. We can now compare the patterns of binding of these Tax/HLA-A2- and M1/HLA-A2-specific Fabs to the previously described panel of HLA-A2 mutants and of Tax and M1 peptide APL to those observed for the panels of Tax- and M1/HLA-A2-specific TCRs. This comparison should reveal, at least for these two experimental systems, whether Abs and TCRs, which are specific for the same peptide/MHC complex, recognize similar or dissimilar components of these complexes. These comparisons will be the first attempt to determine the similarities or distinctions between the Ag recognition patterns of this new class of recombinant MHC-restricted Fabs and TCRs.

### Materials and Methods

#### Peptides

Human T cell leukemia virus type 1 Tax\(^{1-18}\), (LLGYPVVY) (21) and single amino acid substitutions Y5A, P6A, V7R, and Y8A (17, 28, 29) were synthesized by Princeton Biomolecules (Langhorne, PA). Tax variants in which the proline at position 6 was replaced with N-alkylated amino acids N-methyl glycine, N-ethyl glycine, and N-propyl alanine were synthesized by Commonwealth Biotechnologies (Richmond, VA) and were gifts from Dr. B. Baker (University of Notre Dame, South Bend, IN). Influenza virus M1\(^{118–66}\) (GLGFVFTL) (40) and single amino acid substitutions F5A, F7A, and T8A (31, 32) were synthesized by Princeton Biomolecules, as were melanoma Ag recognized by T cells (MART-1\(^{17–26}\); AAGIGILTV) (41), MUC1-D6\(^{13–21}\) (LLLTVLYTV) (34), and gp100\(^{174–182}\) (KTVQGQTVQV) (34). All peptides were HPLC-purified and dissolved in DMSO at 10 mg/ml.

#### Selection of recombinant Fabs with specificity for Tax and M1/HLA-A2

The generation and characterization of a panel of six different Fabs specific for Tax/HLA-A2 have been previously described in detail (37). These phage Abs were originally selected for binding to single-chain MHC-peptide complexes (37, 42) using a large human Fab library containing 3.7 \(\times\) 10\(^6\) different Fab clones (38). DNA sequencing of the six Tax/HLA-A2-specific Fab L and H chain CDR1, -2, and -3 regions showed that each of these Fabs was unique (37). They include members of V\(_{\gamma}\)1, -3, and -4 and V\(_{\delta}\)1, -2, -3 families (37). Identical procedures were used to isolate four phage clones that produce Fabs that specifically bind to the M1/HLA-A2 complex. These four Fabs bound specifically to cell surface M1/HLA-A2 complexes, but not to other peptide/HLA-A2 complexes. These Fabs also specifically bound to soluble M1/HLA-A2 complexes, but not to free M1 peptide in ELISA assays (A. Lev, manuscript in preparation). M1/HLA-A2-specific Fab Abs were expressed and purified as previously described (37). The eluted Fabs were dialyzed twice against PBS (overnight, 4°C) to remove residual imidazole.

#### Generation and expression of HLA-A2 mutant transfectants

Previously, a panel of HLA-A2 mutants (Table I and Fig. 2) was created (26) and transfected into Hmy2,C1R cells (43). In this mutant panel, each of the HLA-A2 amino acids contacted by the Tax-specific A6 TCR was replaced with alanine (or glycine in cases of alanine in the wild-type) by site-directed mutagenesis. All mutant transfectants showed cell surface expression of HLA-A2 at similar levels as wild-type HLA-A2, as detected by the BB7.2 HLA-A2-specific Ab (Fig. 2).

#### Measurement of Tax- and M1/HLA-A2-specific Fabs binding to cell surface peptide/MHC complexes

Hmy2.C1R transfectants (2 \(\times\) 10\(^4\)) were pulsed with 5, 50, or 500 \(\mu\)M peptide in 0.2 ml of X-VIVO 15 (BioWhittaker, Walkersville, MD) serum-free medium in round-bottom, 96-well plates at 37°C overnight. The 500-\(\mu\)M peptide concentration produced reduced cell viability. The cells were then centrifuged, washed in 0.2 ml/well FACS assay medium (PBS, 5% FCS, and 0.2% sodium azide), and resuspended in 10 \(\mu\)l of FACS assay medium. Fabs were added (1.2 \(\mu\)g/2–5 \(\mu\)l) and the cells were incubated for 1 h at 4°C. The cells were centrifuged and washed once with 0.2 ml of FACS assay medium. FITC-conjugated goat anti-human IgG (Fab-specific; Sigma–Aldrich, St. Louis, MO; 50 \(\mu\)l of 1/100 dilution) was added, and the cells were incubated for 1 h at 4°C. The cells were then centrifuged, washed once with FACS assay medium, resuspended in 0.2 ml, and analyzed by flow cytometry using a FACSCalibur (BD Biosciences, Mountain View, CA). Mean fluorescence intensity (MFI) values were calculated using CellQuest software (BD Biosciences). Because the different transfectants express levels of cell surface HLA-A2 that are not identical (Fig. 2), the levels of Tax and M1/HLA-A2 expression detected by Fabs on each transfectant were expressed as a percentage of BB7.2 MFI to normalize for differences in HLA-A2 expression. Comparison of peptide/HLA-A2 Fab binding to peptide/HLA-A2 complexes was measured by ELISA using a large human Fab library containing 3.7 \(\times\) 10\(^6\) different Fab clones (38). DNA sequencing of the six Tax/HLA-A2-specific Fab L and H chain CDR1, -2, and -3 regions showed that each of these Fabs was unique (37). They include members of V\(_{\gamma}\)1, -3, and -4 and V\(_{\delta}\)1, -2, -3 families (37). Identical procedures were used to isolate four phage clones that produce Fabs that specifically bind to the M1/HLA-A2 complex. These four Fabs bound specifically to cell surface M1/HLA-A2 complexes, but not to other peptide/HLA-A2 complexes. These Fabs also specifically bound to soluble M1/HLA-A2 complexes, but not to free M1 peptide in ELISA assays (A. Lev, manuscript in preparation). M1/HLA-A2-specific Fab Abs were expressed and purified as previously described (37). The eluted Fabs were dialyzed twice against PBS (overnight, 4°C) to remove residual imidazole.

#### Table I. Summary of effects of HLA-A2 mutants on recognition by Tax and M1 peptide-specific CTL lines

<table>
<thead>
<tr>
<th>Mutant HLA-A2</th>
<th>Tax</th>
<th>M1</th>
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<tbody>
<tr>
<td>E58A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R65A</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>K66A</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>K68A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A69G</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Q72A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A149G</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A150G</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H151A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Q155A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>T163A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E166A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>W167A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>R170A</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^*\) Negative effects of mutant HLA-A2 molecules are scored as negative (−) if, in the majority of CTL lines tested, they produced a <10-fold reduction in the amount of peptide required to produce 50% of maximum lysis relative to wild-type HLA-A2, ++ if they produced a >100-fold reduction, and + if they produced a 10- to 100-fold reduction in activity by the majority of CTL tested. Results are summarized from Ref. 26 and 27.
detection on the mutants relative to the wild-type cells was expressed as the ratio of mutant normalized MFI to wild-type normalized MFI for each Fab.

**Generation and assay of Tax- and M1/HLA-A2-specific CTL lines**

The generation and specificity of CTL lines specific for Tax and M1/HLA-A2 have been previously described (26, 27). All these CTL populations were shown to lyse HLA-A2<sup>+</sup> targets pulsed with their cognate peptide and did not lyse HLA-A2<sup>+</sup> targets pulsed with other HLA-A2-restricted peptides. Cytotoxicity was quantified by a time-resolved fluorometric assay using HLA-A2 wild-type and mutant transfected Hmy2.C1R cells as target cells, as described previously (26, 27).

**Results**

**Tax- and M1/HLA-A2-specific CTL recognition of HLA-A2 mutants**

Previous studies have characterized recognition of HLA-A2 mutants by panels of Tax- and M1/HLA-A2-specific CTL lines (26, 27). These mutants and their functional effects on these CTL are summarized in Table I. For Tax/HLA-A2-specific CTL, only three mutants on the α1 helix at positions 65, 66, and 69 strongly inhibited CTL recognition, whereas M1/HLA-A2-specific CTL were inhibited by a shared mutant on the α1 helix (K66A), but also by another mutant on the α2 helix (Q155A). Thus, Tax- and M1/HLA-A2-specific CTL recognize some shared as well as distinct components on the surfaces of these peptide/HLA-A2 complexes.

**Specificity of Fabs for Tax and M1/HLA-A2 complexes**

Six previously characterized Fabs were selected for the ability to bind to recombinant single-chain HLA-A2 molecules complexed with the Tax peptide, but not to other HLA-A2-restricted peptides or to the Tax peptide alone (37). To determine whether these Fabs could specifically recognize Tax/HLA-A2 complexes on the surface of HLA-A2<sup>+</sup> cells, A2.1 wild-type transfected Hmy2.C1R cells were pulsed overnight with 50 μM Tax and 50 μM MART-1 peptides, washed, and incubated with the Fabs; Fab binding was detected by flow cytometry. The results demonstrate that each of these Fabs could recognize the A2.1 transfectants pulsed with the Tax peptide, but not with the MART-1 peptide or no peptide (Fig. 1B).

**FIGURE 1.** Specificity of Tax and M1/HLA-A2-specific Fab clones measured by cell surface binding. A, Wild-type A2.1 Hmy2.C1R cells were pulsed with 50 μM Tax (green), 50 μM MART-1 (red), or no peptide (black), then stained with the designated Tax/HLA-A2-selected Fabs and analyzed by flow cytometry. Titration with T3D4 used 0.6 μg (green), 1.2 μg (red), and 2.4 μg (blue) of Fabs/2 × 10<sup>5</sup> cells. B, Wild-type A2.1 Hmy2.C1R cells were pulsed with 50 μM M1 (green), 50 μM Tax (red), or no peptide (black), then stained with the designated M1/HLA-A2-selected Fabs and analyzed by flow cytometry.
Similar results were obtained with 5 μM peptides, although the MFIs were lower (not shown). Thus, the Fabs all have TCR-like specificity for the Tax/HLA-A2 complex. A representative titration with Fab T3D4 (Fig. 1A) demonstrated that 2-fold higher or lower amounts than our standard condition (1.2 μg/2 × 10^5 cells) did not dramatically change the brightness of the signal.

A selection procedure analogous to that used to isolate these Tax/HLA-A2-specific Fabs was employed to select Fabs from the same phage display library that could specifically bind to soluble M1/HLA-A2 complexes, but not to the free M1 peptide. Four of these Fabs were then assayed for the ability to specifically bind to M1/HLA-A2 complexes on the surface of the same HLA-A2 transfectants as those described above. The results (Fig. 1B) demonstrate that each of these Fabs could bind detectably to M1-pulsed cells, but not to cells pulsed with the Tax peptide or no peptide. The levels of M1/HLA-A2 complexes detected on the cell surface were comparable to those detected with the Tax/HLA-A2-specific Fabs.

**Recognition of HLA-A2 mutants by Tax- and M1/HLA-A2-specific Fabs**

The Tax- and M1/HLA-A2-specific Fabs were assayed on the panel of 15 HLA-A2 mutants previously used to characterize MHC recognition by peptide/HLA-A2-specific TCRs (26, 27) (Table I). The levels of cell surface expression of HLA-A2 on each of the mutants detected with the BB7.2 Ab is shown in Fig. 2. The binding of the Fabs to wild-type HLA-A2 pulsed with peptide was compared with each of the mutants by dividing the BB7.2-normalized MFI of binding to peptide-pulsed mutant cells by the BB7.2-normalized MFI of binding to peptide-pulsed, wild-type cells. The y-axis values are the results of this calculation for each Fab.

**FIGURE 2.** Cell surface expression of HLA-A2 on Hmy2.C1R transfectants. Cells expressing wild-type A2.1 or the designated mutants were incubated with BB7.2 Ab and stained with fluorescein anti-mouse IgG. Cell surface expression was quantified as MFI values (y-axis).

**FIGURE 3.** Reactivity of Tax/HLA-A2-specific Fabs with HLA-A2 mutants compared with wild-type transfectants. Cell surface Tax/HLA-A2 complexes were detected on the designated mutant and wild-type transfectants pulsed with 50 μM Tax peptide as described in Materials and Methods. BB7.2-normalized MFI values were calculated for each wild-type and mutant curve, and the MFI of each mutant curve was divided by the MFI of the wild-type curve for each Fab. The y-axis values are the results of this calculation for each Fab.
HLA-A2. If the binding to the mutant is equivalent to that of wild-type HLA-A2, the value of the mutant binding would be 1.0. Negative effects of the mutants on Fab binding would produce values <1, and enhancing effects of the mutants would result in values >1.

The results with the six Tax/HLA-A2 Fabs (Fig. 3) demonstrate that all the Fabs showed >2-fold reduction of binding to at least two of the HLA-A2 mutants, further demonstrating the MHC recognition requirements for binding of these Fabs to Tax/HLA-A2 complexes. The mutants that had the most negative effects on binding of Tax/HLA-A2-specific Fabs were R65A and K66A. Five of six Fabs showed a >3-fold reduction in MFI relative to wild-type for R65A (Fig. 4A), and three of six showed similar reductions with K66A (Fig. 4A). These are two of the three mutants that had significant negative effects on a large panel of Tax/HLA-A2-specific CTL (Table I). The recognition patterns of the Fabs were clearly not identical with the predominant pattern of TCR recognition, because mutants Q72, A150G, H151A, Q155A, A158G, and E166A had >3-fold negative effects on at least one of the Fabs, but had no negative effect on Tax/HLA-A2-specific TCR recognition (Table I).

The four M1/HLA-A2 Fabs were then assayed on this same panel of mutants pulsed with the M1 peptide and were compared with wild-type HLA-A2 (Fig. 5). The results demonstrate that all four Fabs were negatively affected (>3-fold reduction in MFI relative to HLA-A2) by K66A, but the Q155A mutant had no effect on any of these Fabs (Fig. 4B). In addition, the E58A mutant that had no effect on CTL recognition negatively affected recognition by three of the four Fabs (Fig. 4B). Also the W167A and R170A mutants affected recognition by two of the four Fabs (Fig. 4B).

Thus, these M1/HLA-A2-specific Fabs show both similarities and significant differences in HLA-A2 recognition compared with CTL (Table I).

In contrast to these negative effects, all 10 Tax- and M1/HLA-A2-specific Fabs showed enhanced binding to the K68A mutant
Four of six Tax and two of four M1/HLA-A2-specific Fabs bound 1.5-fold more strongly to peptide/K68A than to wild-type peptide/HLA-A2. Two of the Tax/HLA-A2-specific Fabs (T3D3 and T3F2) actually exhibited 2-fold enhanced binding to K68A (Fig. 3). The K68A mutant produced no negative or enhancing effects on any Tax- or M1/HLA-A2-specific CTL line tested (26, 27). These findings suggest that replacement of K68 with alanine removes some structural feature of HLA-A2 that is inhibitory for Fab binding.

Recognition of APL by Tax- and M1/HLA-A2-specific CTL

Previous studies with the Tax/HLA-A2-specific A6 TCR demonstrated that single amino acid substitutions in the Tax peptide that were contacted by the TCR had significant effects on TCR signaling. Substitution of the tyrosine at Y5 with alanine (designated Y5A) and substitution of V7 with arginine (V7R) produced partial agonists (17, 28, 29), whereas P6A and Y8A produced weak antagonists (17). The crystallographic structure of the A6 TCR bound to the P6A/HLA-A2 complex showed a packing defect in the TCR/MHC interface (17). This packing defect could be repaired in a stepwise manner using P6N-alkylated amino acids that resulted in stepwise increases in TCR affinity and activity, thereby converting an antagonist into an agonist peptide (30).

We now use these previously characterized Tax APL to examine a panel of nine additional Tax/HLA-A2-specific CTL lines for their effects on recognition. The results for four of the CTL lines are shown in Fig. 6, and the results for all CTL lines are summarized in Table II. The two CTL lines in the top panels of Fig. 6 represent the extreme opposite patterns of APL recognition observed. Recognition by CTL line JO.23 is negatively affected by all APL, whereas CTL line C2 is only affected by two APL, Y5A and Y8A. Peptide Y5A produced the broadest negative effects on nine of the 10 CTL lines tested (Table II), followed by Y8A, V7R, and

Table II. Summary of effects of Tax APL on Tax/HLA-A2-specific CTL lines

<table>
<thead>
<tr>
<th>CTL Line</th>
<th>Y5A</th>
<th>V7R</th>
<th>Y8A</th>
<th>P6A</th>
<th>P6NMeGly</th>
<th>P6NEtGly</th>
<th>P6NPrAla</th>
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</tr>
<tr>
<td>JO.23</td>
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<tr>
<td>JO.4</td>
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</tbody>
</table>

* Negative effects of Tax APL are scored as negative (−) if they produced a <10-fold reduction in the amount of peptide required to produce 50% of maximum lysis relative to wild-type Tax, and + + if they produced a >100-fold reduction in activity. Results for CTL line RS56 (A6 TCR) are from Refs. 17, 29, and 30.
P6A. Of the seven CTL lines negatively affected by the P6A substitution, recognition of all but one was repaired by the P6N-propyl alanine substitution.

Previous studies using both bulk CTL and CTL clones have shown that almost all M1/HLA-A2-specific CTL are negatively affected by the substitutions F5A and F7A in the M1 peptide, and that the T8A substitution has only modest effects (31, 32). These results can be rationalized by the crystal structure of the M1/HLA-A2 complex in which the phenylalanine side chains at P5 and P7 are available on the surface of the complex to be engaged by the TCR (33). These previous findings are accurately reflected in the results obtained with four of our M1/HLA-A2-specific CTL lines shown in Fig. 7. Recognition by each CTL line was inhibited by F5A and F7A, but not as much by T8A.

Recognition of APL by Tax- and M1/HLA-A2-specific Fabs

Recognition of the four Tax APL with single alanine substitutions by four Tax/HLA-A2-specific Fabs is shown in Fig. 8A. The levels of cell surface expression of HLA-A2 are shown as controls with Ab BB7.2. The P6A substitution had the largest negative effect, producing a >3-fold reduction by each Fab (Fig. 8B). The Y5A substitution that had the strongest effect on CTL recognition also strongly inhibited recognition by three of the four Fabs. The most striking differences between CTL and Fab recognition were observed when comparing recognition of P6A and the P6N-alkylated amino acids (Fig. 9). As noted above, all four Fabs failed to bind well to P6A, and all four also failed to bind more strongly to each of the P6N-alkylated amino acids (Fig. 9). This result is in marked contrast to the finding that six of the seven CTL lines that were inhibited by P6A could be repaired by the P6N-propyl alanine substitution (Table II).

The panel of four M1/HLA-A2-specific Fabs was assayed for binding to the three M1 APL described above: F5A, F7A, and T8A. The F5A substitution only induced a >3-fold reduction in binding by one of the Fabs (M1-A2; Fig. 10). The F7A variant negatively affected binding of two of the Fabs, and the T8A substitution had no negative effect on any of the Fabs. These results

![Graph](https://example.com/graph.png)  
**FIGURE 7.** Recognition of M1 APL by M1/HLA-A2-specific CTL lines. The designated CTL lines were assayed on A2.1-transfected Hmy2.C1R cells pulsed with the indicated concentrations of M1 or M1 APL peptides and assayed for cytotoxicity at an E:T cell ratio of 2.5:1.

![Graph](https://example.com/graph.png)  
**FIGURE 8.** Recognition of Tax APL by Tax/HLA-A2-specific Fabs. A, A2.1-transfected Hmy2.C1R cells were incubated with 50 μM Tax or the indicated Tax APL and then stained with Tax/HLA-A2-specific Fabs or BB7.2. The y-axis numbers are the MFI for the APL peptides divided by the MFI for the wild-type Tax peptide. B, The number of Fabs showing >3-fold reduction in APL MFI relative to the wild-type Tax peptide.
are significantly different from the observations with the CTL lines in Fig. 7 and those reported previously (31, 32).

**Summary of recognition of peptide/HLA-A2 complexes by Tax/HLA-A2- and M1/HLA-A2-specific TCRs and Fabs**

A graphical summary of the similarities and differences in TCR and Fab recognition of the Tax/HLA-A2 and M1/HLA-A2 complexes is provided in Fig. 11. The only common element for both these classes of immunoreceptors was the ability of the K66A mutation to negatively affect recognition by the majority of each of the panels of TCRs and Fabs. In general, the recognition patterns of the Tax/HLA-A2-specific TCRs and Fabs were more similar to each other than were those of the M1/HLA-A2-specific TCRs and Fabs.

**Discussion**

In the present study we compared the reactivity patterns of a panel of Tax- and M1/HLA-A2-specific Fabs with those of a panel of HLA-A2 mutations and APL that had previously been used to characterize TCR recognition (17, 26–32). The results indicate both striking similarities and important differences between Fab and TCR recognition of peptide and MHC components of the Tax and M1/HLA-A2 complexes. Specifically, our results showed that the mutants R65A and K66A that produced the focused negative effects on Tax/HLA-A2-specific TCR recognition also were the most disruptive for Tax/HLA-A2-specific Fab recognition. A similar pattern was observed for the M1/HLA-A2-specific Fabs; one of the two mutants (K66A) that was the most disruptive for TCR recognition also negatively affected binding by all four Fabs. For both Tax- and M1/HLA-A2-specific Fabs, binding was disrupted by multiple mutants that had no negative effect on TCR recognition. Also, one of the most obvious differences was the negative effect of the E58A mutant on three of four M1/HLA-A2-specific Fabs, with no effect on any HLA-A2-restricted TCR (27). Overall, these results indicate that MHC-restricted Fabs and TCRs bind to overlapping, but nonidentical, structural components of the MHC.

The enhanced binding of all the Tax- and M1/HLA-A2-specific Fabs to the K68A mutant was not observed for any of the other HLA-A2 mutants. Such enhanced recognition of K68A was not observed for recognition by any Tax or M1/HLA-A2-specific CTL line tested (26, 27). The replacement of the positively charged lysine side chain at position 68 with alanine on the α1 helix could make the surface of the MHC molecule more open and hydrophobic. Such a change appears to remove some negative effect(s) in the Fab/peptide/HLA-A2 interface. This observation suggests that the ligand binding sites of these Fabs could be engineered to accommodate the interfering MHC structures in the interface, thereby producing Fabs that exhibit higher affinity binding to the peptide/MHC complexes. Such higher affinity Abs could have enhanced potential in immunodiagnostic and immunotherapeutic applications.

Comparison of the fine specificities of TCRs and Fabs for recognition of the peptide components of the Tax and M1/HLA-A2 complexes also demonstrated both striking similarities and differences in patterns of recognition. For the Tax peptide, those single amino acid substitution APLs that had significant effects on TCR interaction also affected Fab binding. However, when one Tax APL was examined in more detail, clear differences emerged between TCRs and Fabs. Substitution of P6 with alanine increased the cavity volume of the A6 TCR/peptide/HLA-A2 interface by 38 Å (17). A water molecule is found in this enlarged cavity. These structural changes result in a reduction of A6 TCR affinity by >100-fold (30). Tax variants in which P6 was replaced with N-alkylated amino acids that were intended to remove the water molecule repair the packing defect of the P6A APL and result in recovery of recognition by the A6 TCR (30). For recognition by other Tax-specific TCRs, six of the seven TCRs that were negatively affected by the P6A substitution were repaired by the P6N-propyl alanine Tax variant (Table II). In sharp contrast, all four Tax/HLA-A2-specific Fabs were negatively affected by the P6A substitution, and no increase in binding by the P6N-propyl alanine Tax variant was observed (Fig. 9). What structural attributes of the interfaces...
between peptide/MHC complexes and TCRs vs Fabs could account for these differences? When the Tax-specific A6 and B7 TCRs bind to the Tax/HLA-A2 complexes, the Tax peptide is “squished,” with the side chain of proline at P6 being pressed down to achieve a close fit in the interface (14, 16). One possibility is that the Fabs do not cause comparable “squishing” of the P6 side chain. If this were correct, then the P6A substitution may not produce any packing defects and insertion of destabilizing water molecules, but may simply remove some key contacts between the Fab and the peptide.

The M1 APL also showed marked differences between the Fabs and TCRs. The F5A M1 variant that had very negative effects on CTL recognition only diminished binding for one of the four Fabs. The phenylalanine side chain at P5 of the M1 peptide points toward the /H9251 helix of HLA-A2, where it makes contact with Q155 and L156 and is also available for contact by TCRs (33). Substitution of alanine for phenylalanine at this position apparently changes the conformation of the peptide/MHC complex that is recognized by most M1/HLA-A2-specific TCRs, but not most Fabs, highlighting the differences in the recognition of this complex by these TCRs and Fabs. A structural explanation for these observed differences will require three-dimensional structures for both TCRs and Fabs bound to the native peptide and the APL complexed with HLA-A2. While other reports have emphasized similarities between the peptide fine specificity of MHC-restricted Abs and TCRs (8, 10–12, 44, 45), it should be noted that all these other studies have involved comparisons of very few TCRs and mAbs that recognize the same individual peptide/MHC complexes. Our results comparing Fab and TCR binding to the Tax and M1/HLA-A2 complexes are somewhat different from those obtained by Messaoudi et al. (44) for an OVA peptide presented by H-2Kb. In that study two different mAbs and one TCR (OT-1) that recognize the OVA/Kb complex were compared for recognition of a panel of single amino acid substitutions of the OVA peptide and a series of naturally occurring Kb mutants, each of which contained multiple amino acid substitutions (except Kb5). The results indicated that both mAbs bound the right side of the /H9251 and /H9252 helix and overlapped with the TCR /-chain footprint on the Kb molecule. In contrast, binding of all but one of our peptide/HLA-A2-specific Fabs was inhibited by at least one substitution on the left side of the /-helix.

One possible reason for the observed differences in peptide/MHC complex recognition between TCRs and these Fabs is that the Fabs are selected from a phage display library and have not been derived from Ag-reactive B cells that have undergone clonal selection. However, all these Fabs are encoded by human germline V genes derived from B cells of the donors of the phage display library (38). The observed affinities of the Tax/HLA-A2-specific Fabs T3F2 and T3E3 are in the 25–30 nM range (37), which is typical for naturally occurring Fabs (46). The relatively high frequency with which HLA-A2-restricted Fabs that are specific for two epitopes derived from telomerase (34) and Tax (37) have been
selected suggests that these molecules may be present at relatively high frequencies in the Ab repertoires of the B cells used to produce the phage library. The only unusual feature of the Fabs is that they could be the product of heavy and light chains that did not occur in any B cell used to generate the library.

TCRs bind to peptide/MHC complexes in a diagonal mode, with the α-chain oriented over the amino-terminal segment of the peptide and the β-chain over the carboxyl-terminal end (13–23). No such structural information currently exists for MHC-restrictedAbs. It is possible that the observed differences in fine specificity for both peptide and MHC components between our panels of TCRs and Fabs reflect differences in the way these two classes of immunoreceptors have solved the problem of specific recognition of complexes of peptide and MHC molecules. These differences may reflect the more plastic nature of the Ab-Ag binding site that can undergo ligand-induced domain shifts (I) that have not been generally observed in TCRs. Resolution of this issue could be answered by production of crystallographic structures of these MHC-restricted Fabs bound to their cognate peptide/MHC complexes and comparison with existing TCR/peptide/MHC structures.

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References


