The Study of High-Affinity TCRs Reveals Duality in T Cell Recognition of Antigen: Specificity and Degeneracy[1]

David L. Donermeyer,[2]* K. Scott Weber,[2]*† David M. Kranz,† and Paul M. Allen[3]*

TCRs exhibit a high degree of Ag specificity, even though their affinity for the peptide/MHC ligand is in the micromolar range. To explore how Ag specificity is achieved, we studied murine T cells expressing high-affinity TCRs engineered by in vitro evolution for binding to hemoglobin peptide/class II complex (Hb/I-E^k). These TCRs were shown previously to maintain Ag specificity, despite having up to 800-fold higher affinity. We compared the response of the high-affinity TCRs and the low-affinity 3.L2 TCR toward a comprehensive set of peptides containing single substitutions at each TCR contact residue. This specificity analysis revealed that the increase in affinity resulted in a dramatic increase in the number of stimulatory peptides. The apparent discrepancy between observed degeneracy in the recognition of single amino acid-substituted Hb peptides and overall Ag specificity of the high-affinity TCRs was examined by generating chimeric peptides between the stimulatory Hb and nonstimulatory moth cytochrome *c* peptides. These experiments showed that MHC anchor residues significantly affected TCR recognition of peptide. The high-affinity TCRs allowed us to estimate the affinity, in the millimolar range, of immunologically relevant interactions of the TCR with peptide/MHC ligands that were previously unmeasurable because of their weak nature. Thus, through the study of high-affinity TCRs, we demonstrated that a TCR is more tolerant of single TCR contact residue substitutions than other peptide changes, revealing that recognition of Ag by T cells can exhibit both specificity and degeneracy. ***The Journal of Immunology,* 2006, 177: 6911–6919.**

The recognition of peptide/MHC (pMHC)[4] complexes by the TCR is a central event in the adaptive immune response. The TCR is an intricate and enigmatic receptor in that it exhibits a high degree of specificity, despite having a micromolar affinity for agonist pMHC ligands expressed on the surface of APCs (1, 2). It has not been established how the TCR is able to maintain the observed specificity with these low-affinity interactions. Several theories have been proposed that focus on multistep signal transduction pathways initiated by the TCR recognition of pMHC. These complex signaling pathways are proposed to permit kinetic proofreading that allows for a T cell to recognize Ag with a high degree of specificity, yet with a relatively low-affinity TCR (3, 4).

The high degree of Ag specificity in T cell recognition of Ag is a sine qua non of the immune system. The early studies characterizing the specificity of CD4$^+$ T cells used model Ags, such as pigeon cytochrome *c* or hen egg-white lysozyme (5, 6). Purified proteins from different animal species of cytochrome or lysozyme with known sequences were then tested for their ability to cross-stimulate T cells. CD8$^+$ T cell responses to different influenza viral subtypes were examined in a similar manner (7, 8). The discovery that T cell Ags could be represented by peptides and the ability to generate peptides synthetically greatly facilitated the exploration of the specificity of T cell recognition of Ag. Single amino acid substitutions in epitopes were produced and tested for their ability to bind to MHC molecules and stimulate T cells (9–14). From these studies, exquisite side-chain specificity at TCR contact residues was shown, in that one or very few other side chains could be recognized at key positions. An outgrowth of these studies was the discovery of altered peptide ligands (APLs). APLs represent a class of TCR ligands, which, unlike full agonists, stimulate some, but not all T cell functions (reviewed in Ref. 15). In general, they are produced by making a single amino acid substitution at a TCR contact residue. The study of APLs revealed that there is an essential flexibility in the T cell recognition of Ag. For T cells to develop in the thymus, their TCR needs to interact with endogenous self peptide APLs. APLs have also been shown to be able to be generated by changes in the peptide's MHC anchor residues. The interactions between a TCR and APL/MHC are much weaker than with agonist/MHC ligands, making it difficult to study the binding affinity and kinetics of APLs. However, there is still a high degree of specificity in the recognition of APLs by TCRs.

T cell specificity has also been studied extensively in the exploration of the concept of molecular mimicry and the initiation of autoimmune responses (16). There are now multiple examples of two distinct peptides stimulating the same T cell. In many of these cases, there are some conserved structural features (17–24), but there are other pairs of stimulatory peptides in which there is no obvious sequence homology (25–29). The screening of combinatorial peptide libraries has also shown that there can be multiple stimulatory peptides for the same T cell (30–32). Thus, there are different molecular solutions to generate stimulatory ligands for the same TCR. Clearly, the phenomenon of two apparently distinct peptides stimulating the same T cell has been established, showing there is degeneracy in the recognition of Ag by T cells. Despite this

*Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110; and †Department of Biochemistry, University of Illinois, Urbana, IL 61801

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[2] D.L.D. and K.S.W. contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. Paul M. Allen, Washington University School of Medicine, Department of Pathology and Immunology, 660 South Euclid Avenue, Campus Box 8118, St. Louis, MO 63110. E-mail address: pallen@wustl.edu

[4] Abbreviations used in this paper: pMHC, peptide/MHC; APL, altered peptide ligand; Hb, hemoglobin; MCC, moth cytochrome *c*; SPR, surface plasmon resonance.

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recognized degeneracy in Ag recognition, there is still little evidence supporting the concept that molecular mimicry is a major contributing mechanism in the initiation of autoimmunity (33). Also, hampering the study of T cell recognition of Ag is that there is no consensus definition in the field for degeneracy or specificity. Therefore, the TCR appears to have different degrees of specificity, depending upon how the specificity is analyzed and defined. Given our current level of understanding, it is conceptually difficult to propose how a TCR can exhibit these apparently contradictory degrees of specificity. It is this question that is the focus of this study. Our examination of the concept of degeneracy refers exclusively to the case in which there are structurally similar peptide Ags (as would be the case in molecular mimicry), and it does not address the question of Ags that are structurally very different.

The 3.L2 model Ag system, specific for hemoglobin (Hb) (3) peptide presented by I-E^k, has been used to define APLs, describe and characterize the immunological synapse, and explore the nature of pMHC ligands involved in T cell development and function (34–38). Previously, we identified and analyzed the affinity and binding kinetics of a series of APLs recognized by 3.L2, which represent a continuum of activities ranging from agonists to antagonists to null ligands (35). Transgenic mice expressing each of these ligands were generated and crossed to 3.L2 TCR transgenic mice, revealing a correlation of the TCR-binding activity with the biological activity of the ligands (36, 37). However, binding affinities could not be determined for all of the biologically active APLs because the interactions were too weak for detection by surface plasmon resonance (SPR). Similar problems with measurements of binding affinities by SPR have been observed recently for an autoimmune TCR:myelin basic protein/HLA-DR2 interaction (39).

We have developed a strategy for probing the specificity of the TCR:pMHC interaction using high-affinity TCRs. Our rationale was that the normal low affinity of the TCR:pMHC interaction significantly limits our ability to probe the pMHC recognition of the TCR. Thus, for most T cells, there may only be at best a 10- to 30-fold affinity window in which to study a TCR interaction(s) with pMHC; ligands that fall below the threshold for SPR measurements would all be categorized as negative even though they may interact with different affinities. By significantly increasing the TCR affinity window, we would greatly improve our ability to assess TCR recognition of different ligands. This would provide a much more accurate picture of the specificity of a TCR for related and unrelated ligands. Because high-affinity TCRs are eliminated during negative selection in the thymus, and thus do not exist in the mature T cell repertoire, we used yeast surface display and in vitro evolution (40–42) to generate a series of high-affinity 3.L2 TCRs evolved for up to an 800-fold increase in affinity for Hb/I-E^k (43). These TCRs contain mutations in the CDR loops of the TCRα and TCRβ chains, which comprise the pMHC-binding interface. T cells transfected with the high-affinity TCRs maintained their Ag specificity, being stimulated only by Hb and not by the control peptide, moth cytochrome *c* (MCC), or APCs alone. Surprisingly, they did not have any increased sensitivity to the wild-type Hb Ag, revealing an affinity activation threshold above which affinity increases do not yield enhanced activation (43). Thus, T cells transfected with high-affinity TCRs were functional and remained Ag specific.

In this study, we used these high-affinity TCRs to probe the specificity of Ag recognition by T cells. The large increase in affinity greatly expanded the affinity window with which we could interrogate Ag specificity. These studies revealed that a TCR can exhibit two distinct patterns of recognition: degeneracy when single amino acid substitutions of solvent-exposed residues are made and specificity when changes are made in both the MHC and TCR contact residues of the peptide.

Materials and Methods

T cell hybridoma generation

T cell hybridomas were generated by the following: 1) cotransfecting full-length M4 TCRα (CDR3 mutations: S98αP, N101αS, Y102αR, and K103αN) and wild-type 3.L2β chain to generate the M4 T cell; 2) cotransfecting M4 TCRα (CDR mutations S98αP, N101αS, Y102αR, and K103αN) and the TCRβ chain containing the CDR3 mutation Q105βR to generate the M14 T cell; and 3) cotransfecting M15 TCRα (CDR mutations K25αE, T28αS, S98αP, N101αS, Y102αR, and K103αN) and M15 TCRβ (CDR3 mutation Q105βR) chains to generate the M15 T cell. These α- and β-chains were cloned in pcDNAzeo and pcDNAneo, respectively, and transfected into a T cell hybridoma that does not express TCRα or TCRβ, which had been transfected with CD4 ($58\alpha^-\beta^-CD4^+$) (44). Cells were selected in zeocin and G418 and subcloned, and individual clones were functionally tested for response to Hb. Several strongly reactive clones were obtained and were further selected for TCR expression levels (Vβ8.3) similar to that of the previously generated 3.L2 hybridoma that expresses full-length 3.L2 TCRα and TCRβ chains in the same cell line (44). Several clones were obtained and tested, and identical results were observed.

T cell hybridoma assays

Stimulation of the T cell hybridomas was determined by IL-2 production using a standard T cell hybridoma assay. A total of 5×10^5 T cell hybridoma cells was cocultured with 3×10^5 CH27 cells and various doses of Hb peptide for 24 h. Supernatants were removed, and the level of IL-2 produced was quantified by a bioassay using the IL-2-reactive cell, CTLL. A total of 5×10^3 CTLL cells was added to 100 μl of supernatant; 18–24 h later, each well was pulsed with 0.4 μCi of [^{3}H]TdR, and the level of incorporation was ascertained 24 h later. EC_{50} values were calculated from the concentration of Hb that stimulated 50% of the maximal response. Relative activities of the individually substituted peptides were calculated by determining the EC_{50} for each peptide, rounding them to the nearest decade value, and comparing them with the EC_{50} value for 3.L2. Each cell line was tested three to four separate times, and a representative assay is shown.

Peptide synthesis

Peptides were synthesized using standard Fmoc chemistry on a Rainin Symphony/Multiplex multiple peptide synthesizer (Protein Technologies). All peptides were >95% pure by analytical reverse-phase HPLC on a C_{18} column (Vydac), and molecular mass and purity were confirmed by MALDI mass spectrometry (Washington University Mass Spectrometry Facility). Representative peptides were purified and tested against their crude counterparts, and no difference was seen in the T cell hybridoma responses.

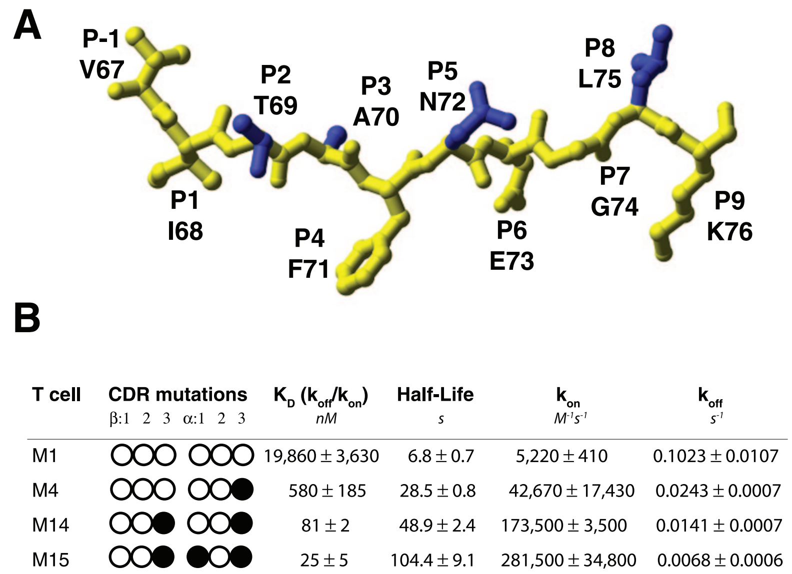
Peptide structural comparisons

The coordinates of the crystal structures of Hb/I-E^k (1FNG) and MCC/I-E^k (1KT2) were used to compare the conformations of the peptides, as previously described (43). The pMHC molecules were aligned by their I-Eα chains using the Insight II suite of programs (Molecular Simulations). Representations of the peptides were generated showing the P1 to P9 residues of the peptides using Insight II.

Results

M4 and M15 have broadened recognition of the P5 of Hb

To examine the effects of high affinity of the TCR recognition of Hb (Fig. 1*A*), we examined T cells expressing either the wild-type 3.L2 TCR (K_D value = 12 μM), the high-affinity M4 TCR (580 nM), or the highest affinity M15 TCR (25 nM). The M4 TCR contains four mutations in the CDR3α chain, and the M15 TCR contains three additional mutations, two in the CDR1α and one in the CDR3β. The increased affinities of the M4 and M15 for Hb/I-E^k are due to both faster k_{on} rates and slower k_{off} (Fig. 1*B*). We have shown previously that the centrally located Asn P5 side chain (residue 72) of Hb is a dominant TCR contact residue (Fig. 1*A*) and that the 3.L2 TCR can interact productively with a continuum of P5 APLs. These include the weak agonist T72, the antagonists I72



T cell	CDR mutations β:1 2 3 α:1 2 3	K_D (k_{off}/k_{on}) nM	Half-Life s	k_{on} $M^{-1}s^{-1}$	k_{off} s^{-1}
M1	○○○ ○○○	19,860 ± 3,630	6.8 ± 0.7	5,220 ± 410	0.1023 ± 0.0107
M4	○○○ ○○●	580 ± 185	28.5 ± 0.8	42,670 ± 17,430	0.0243 ± 0.0007
M14	○○● ○○●	81 ± 2	48.9 ± 2.4	173,500 ± 3,500	0.0141 ± 0.0007
M15	○○● ●○●	25 ± 5	104.4 ± 9.1	281,500 ± 34,800	0.0068 ± 0.0006

FIGURE 1. Hb peptide residues and location of CDR mutations in high-affinity 3.L2 TCR mutants. *A*, Structure of Hb peptide (ITAFNEGLK) showing in blue the location of the four surface-exposed residues P2(T), P3(A), P5(N), and P8(L). *B*, 3.L2 M1 (unmutated CDRs) and three higher affinity mutants (M4, M14, and M15) with the location of their CDR mutations and respective K_D, $t_{1/2}$, k_{on}, and k_{off} values (43). The CDRβ1,2,3 and CDRα1,2,3 are designated from *left* to *right* by circles: ○, indicate that the CDR is not mutated; ●, indicate mutations in the CDRβ or CDRα, respectively. The amino acid mutations in these regions are the same as previously published (43).

and A72, and a positively selecting ligand Q72. (i.e., T72 refers to a substituted Hb peptide in which a Thr has been substituted at the 72 (P5) and other peptides are similarly designated). No activity has been identified for the null ligand E72 (35).

To explore what changes the higher affinity of M4 and M15 had on the recognition of Hb, we generated a complete set of Hb variant peptides, each containing 1 of the 20 naturally occurring amino acids at P5, including the wild-type Asn. Comprehensive substitution of TCR contact residues, P2, P3, P5, and P8, in Hb and MCC has been previously examined and shown not to affect binding of the peptides to I-E^k (45, 46). The crystal structure of Hb/I-E^k and MCC/I-E^k supported the binding results, in that these side chains are completely solvent accessible and do not make any contacts with the I-E^k molecule (47, 48). Thus, in this type of analysis, the effects of the single amino acid substitutions can be ascribed to alterations in the interactions with the TCR, and not to interactions with I-E^k, and thus the number of pMHC complexes. Each of these peptides was then tested for their agonist activity by their ability to stimulate 3.L2, M4, and M15 T cells and grouped according to the chemical nature of the side chains (polar, aliphatic, or charged). Cys residues were replaced by Aib (amino isobutyric acid) to avoid dimerization issues, and were classified as aliphatic.

As we had observed previously (49), there was remarkable specificity at P5 for 3.L2 with only the wild-type Asn residue being fully stimulatory and Val, Thr, and Cys (Aib) being stimulatory, but each being 10- to 100-fold weaker (Fig. 2). With M4 T cells, there was a dramatic increase in the number of stimulatory peptides. A total of 11 of 20 peptides stimulated the M4 cells, with Ala, Asn, Ile, Cys (Aib), Leu, Met, Ser, Thr, and Val being full agonists or 10-fold weaker stimulators than wild type, and Gly and Asp being 100- to 1000-fold weaker stimulators. These changes in agonist activity appeared to follow the previous defined activity patterns for 3.L2 with the weaker agonist becoming stronger (i.e., Thr), and APLs becoming agonists (i.e., Ala, Ile). For the highest affinity T cell, M15, the activity pattern of the peptides further changed with all of the M4 agonists becoming as strong as wild-type Asn (Fig. 2). The merging of the activity profiles is reflective of our previous demonstration of an activity threshold above which there was not an increase in T cell activation. The only residue that had no activity for M4, but was a partial agonist for M15, was Gln. The eight residues that were not stimulatory for M15 had some biochemical features in common, being the large, hydrophobic, or charged residues (Pro, Phe, Trp, Tyr, Glu, His, Lys, and Arg). Given that the binding affinity of the 3.L2:Hb/I-E^k interaction has a K_D of 20 μM (as measured for M1, the corresponding single-chain TCR for 3.L2) (43) and yields full agonist activity, we can conclude that the affinity of M15 for these large hydrophobic or charged residues must be at least 800-fold lower than the M15: Hb/I-E^k interaction (K_D = 25 nM). Thus, despite the increase in reactivity with P5 variants, there remains a considerable impact of the P5 side chain on TCR binding.

Of particular interest are the changes in the activities of the six ligands for which we have defined a continuum of activities (36, 37). Four of these ligands (Thr, Ile, Ala, Gln) increased their activities, but kept their activities relative to each other. Thus, the strongest APLs all became agonists with M4, with Gln remaining negative. With M15 these ligands all became agonists, including Gln. Importantly, our null ligand, Glu, remained negative in all of these studies. Overall, these studies involving the recognition of the P5 residue show that the higher affinity TCRs have a significant broadening of the number of side chains that yield biological activity and that the changes in activities of the peptides correlate to their previously identified function.

M15 has broadened recognition of Hb at all TCR contact positions

Based on the findings with the characterization of P5, we wanted to perform a comprehensive analysis of the recognition of Hb by the three T cells. We individually tested all 20 aa at each of the four TCR contact residues, P2, P3, P5, and P8 (Fig. 1*A*). Each of the peptides was tested over a large concentration range for the ability to stimulate the M4, M15, and 3.L2 T cell hybridomas, and the results are summarized in Fig. 3. The results clearly show that M4 and M15 are stimulated by many more peptides than 3.L2 cells. Recognition of P2 × 3.L2 is the most degenerate of the four TCR contact residues, in that eight different residues can stimulate. M4 and M15 showed maximal degeneracy at the P2 position with 19 and all 20 side chains being recognized, respectively. At P2 there was the same trend as observed at P5, that the activity of the allowable side chains increased between M4 and M15. The recognition of the P3 position was similar to P2, in that five different

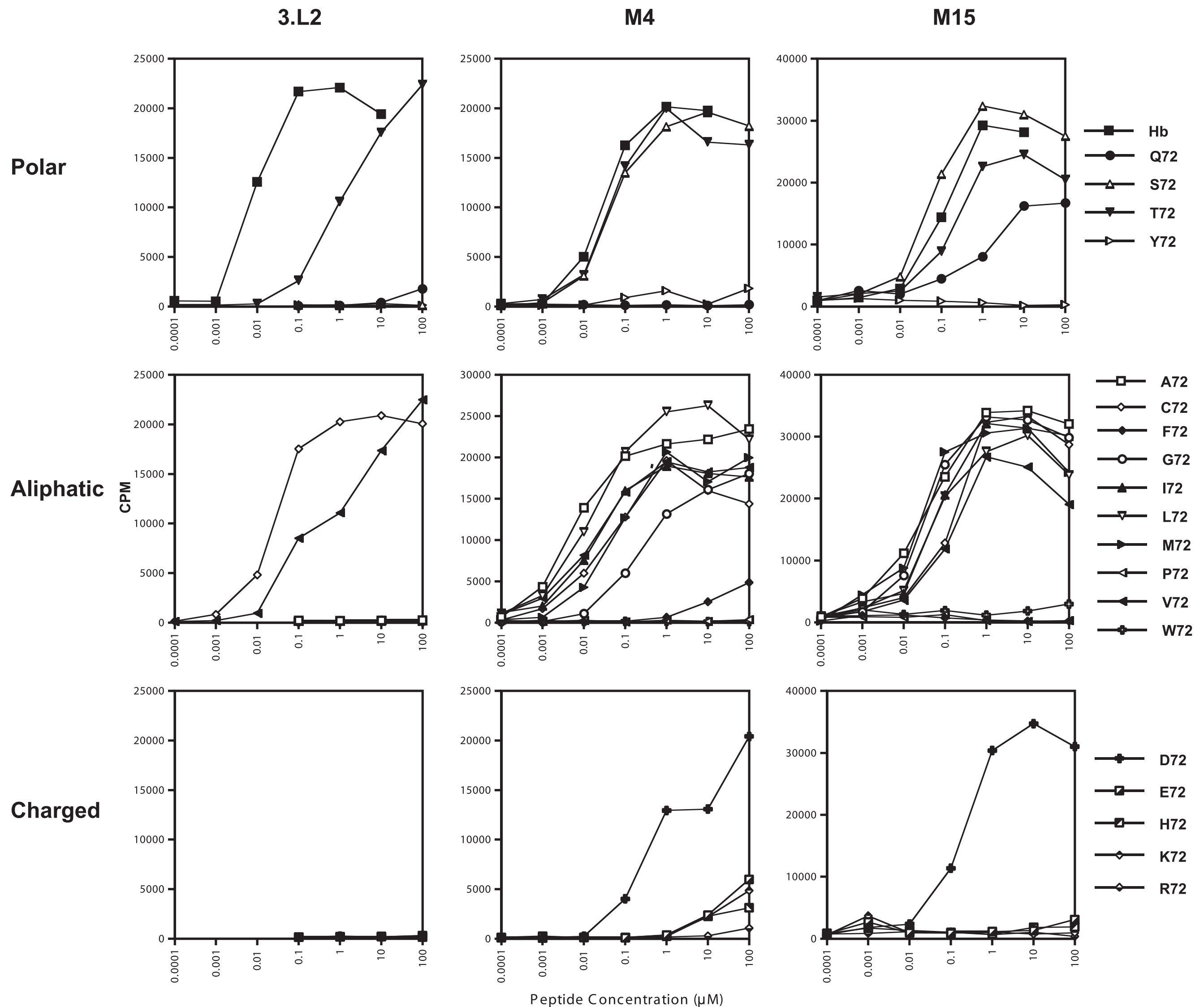


FIGURE 2. Broadened local specificity at the main TCR contact residue (P5) of affinity clones M4 and M15 compared with wild-type 3.L2. $CD4^+$ T cell hybridomas expressing wild-type 3.L2, M4, and M15 TCRs were stimulated with the indicated peptide concentrations of Hb(Hb(64-76)) or APLs (single amino acid substitutions) using CH27 cells as APCs. APLs shown are all at the main TCR contact residue (P5) and are divided into rows (polar, aliphatic, or charged) dependent upon the nature of the residue at position P5. They are designated using the one-letter amino acid code for the residue. The Hb wild-type residue at position P5 is Asn. Aminobutyric acid residues were substituted for cysteine residues, to avoid the dimerization concerns, and are listed as C. The level of T cell stimulation was determined using a bioassay for IL-2. The cpm values of $[^3H]$TdR incorporation into CTLL-2 cells represent the mean of triplicate values of a representative experiment.

amino acids were recognized by 3.L2, and that increased to 18 for M4 and M15. Thus, at the P2 and P3 positions, little side chain specificity was observable. There was some trend in that the charged residues (Lys, Arg, Glu, and Asp) were the least active.

In contrast, there was a much higher degree of specificity observed in the recognition at the P8 position. For 3.L2, only the wild-type residue Leu was recognized. For M4, three additional side chains were allowable, all of which were aliphatic (Val, Ile, and Cys (Aib)), demonstrating a high degree of recognition. With M15 there was a significant increase in the number of allowable residues at P8 to 13. There were some, but not complete, chemical correlates in the recognizable side chains, with all but the largest aliphatic (Phe, Trp) side chains causing stimulation. Two small polar (Ser, Thr) and 2 positively charged (Lys, Arg) amino acids also simulated M15. Collectively, these data show a remarkable broadening of the recognition of Hb with the higher affinity TCRs M4 and M15. For 3.L2, 14 single amino acid substitutions of the Hb peptide are recognized, which increased to 47 for M4 and 59 for M15. Furthermore, the relative order of specificity ($P8 > P5 > P2 > P3$) was maintained for 3.L2, M4, and M15. This result is consistent with the notion that the high-affinity TCRs have maintained the same general docking pattern as the wild-type TCR. Thus, within the context of the Hb peptide and analysis by peptides containing single amino acid substitutions in solvent-exposed residues, the result of the increased affinity of the TCR is increased degeneracy in the recognition of Hb as determined by T cell activity.

Increased degeneracy of Hb recognition directly correlates with increasing affinity

The findings above with M4 and M15 revealed a correlation between increased affinity of the TCR:pMHC interaction and the increased degeneracy. To test this correlation further, we performed a peptide specificity analysis of the P8 position with T cells

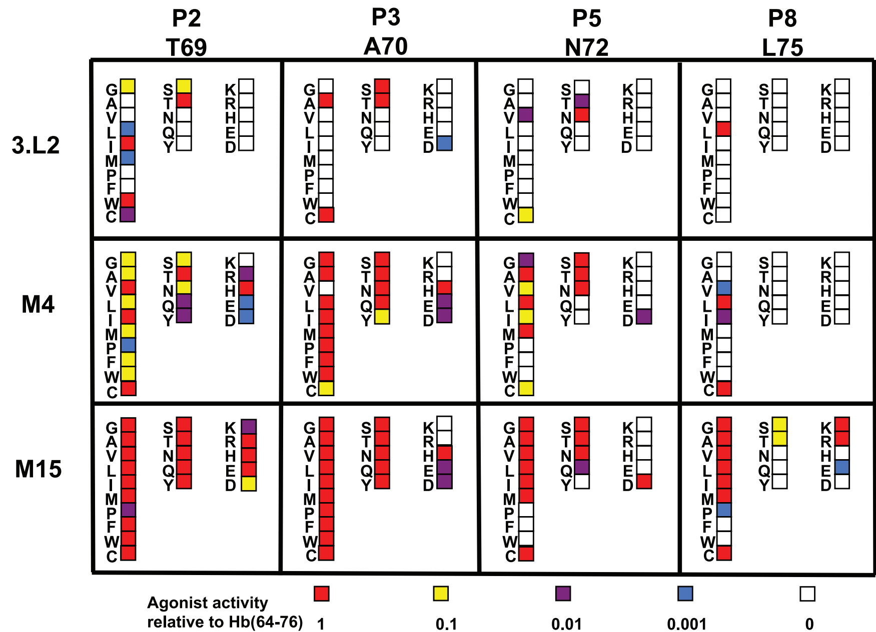


FIGURE 3. Activity of Hb peptides with substitutions at the four surface-exposed residues. Local specificity of 3.L2, M4, and M15 was determined by testing each cell line with Hb peptides containing a single amino acid substitution at each of the four exposed peptide positions. All other 19 naturally occurring amino acid residues were tested for a total of 76 peptides. The peptides are indicated by the substituted position using one-letter amino acid codes and group according to polar, aliphatic, and charged residues. Each peptide was tested as in Fig. 2 over a wide concentration range and repeated two to five times with each T cell hybridoma. EC_{50} values were calculated in relation to the response to Hb wild-type peptide. The values were rounded to the nearest decade and color coded. Unfilled boxes indicate that a peptide did not stimulate at concentrations up to 100 μM. For example, the G at P2 is the peptide Hb(64-76)G69, which had an EC_{50} value 10-fold weaker for 3.L2 and M4 cells compared with the M15 Hb response.

expressing the M14 TCR, whose affinity (81 nM) is between M4 (580 nM) and M15 (25 nM) (Fig. 1). The direct relationship between the affinity of the TCR:pMHC interaction and the number of ligands recognized could be clearly illustrated by the response to three substitutions of the wild-type (Leu) residue at P8 (Fig. 4). The 3.L2 T cells did not recognize Ile, Ala, or Lys at P8. The next higher affinity T cell, M4, responded to Ile, but not the other two. The increased affinity of M14 resulted in Ala now stimulating at higher concentrations, but Lys still being nonstimulatory. For the highest affinity, M15, all three peptides stimulated as well as Hb. Thus, by analyzing a third high-affinity TCR, we were able to more thoroughly demonstrate directly the relationship between increased affinity and a greater number of side chains recognized.

Energetic effects of the recognition of APLs by high-affinity TCRs

The demonstration of increased degeneracy by the higher affinity TCRs (supra vide) provides a unique opportunity to explore the energetics of the recognition of biologically active Hb APLs, the nature of which for 3.L2 were too weak to analyze previously. Gln^{72}, when expressed in vivo, resulted in enhanced positive selection of 3.L2 T cells (36). Gln^{72} did not stimulate 3.L2 or M4 T cells, but was a weak agonist for M15 (Fig. 2). A peptide residue substitution that is an agonist for M15, but does not stimulate M4 or 3.L2, has at least an 800-fold energetic effect upon the binding interaction. The 3.L2:Hb/I-E^k interaction has a K_D of 20 μM (as measured for M1, the corresponding single-chain TCR for 3.L2) (43). Therefore, if we assume that the differences in affinity (of 3.L2 for Gln^{72}/I-E^k and M15 for Gln^{72}/I-E^k) are proportional, we can estimate that the affinity of the 3.L2:Gln^{72}/I-E^k interaction would be in the range of 12 mM. Glu^{72} demonstrates that these weak interactions still have a degree of specificity. Glu^{72} has no activity in vivo (36) and was not stimulatory for 3.L2, M4, or M15 (Fig. 2). We cannot assign an exact energetic impact for it, but it would be at least 800- to 1000-fold weaker than the 3.L2:Hb/I-E^k interaction. These findings indicate that TCR:pMHC interactions that are involved in positive selection, which have a significant degree of specificity, occur in the millimolar affinity range (again assuming a direct correlation between reduction in binding energies for the M15 interactions and the 3.L2 interactions). Similarly, the affinity of the interactions between a TCR and antagonist/MHC has been examined; however, they are close to the limits of reliable SPR detection. For example, Ile^{72} is an antagonist for 3.L2 and can provide enough binding energy to replace CD4 (36). It is an agonist for M4 and M15 (Fig. 2), giving it an energetic contribution of

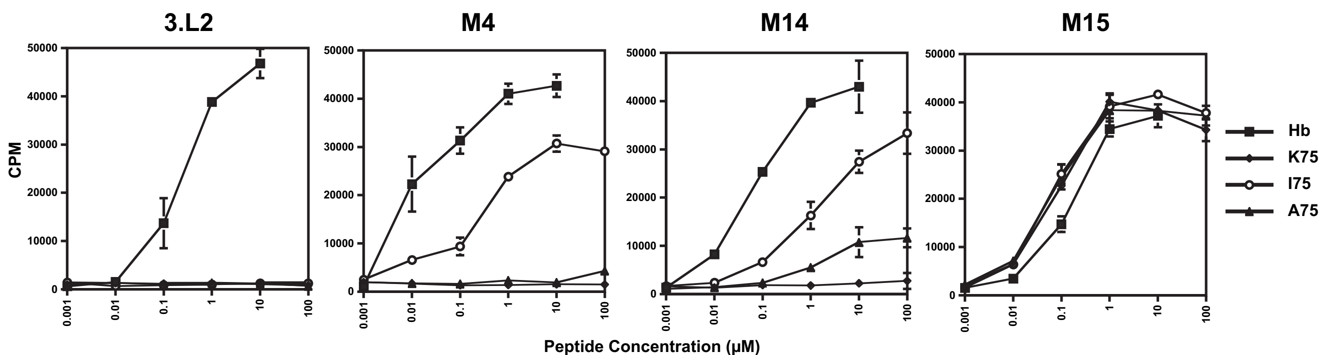


FIGURE 4. Degeneracy in the recognition of Hb correlates with increased affinity. $CD4^+$ T cell hybridomas expressing 3.L2, M4, M14, and M15 TCRs were stimulated with the indicated peptide concentrations of Hb or APLs of Hb at the P8 (75) position using CH27 as APC. The APLs are designated as described in Fig. 2. The level of T cell stimulation was determined using a bioassay for IL-2. The cpm values of [^{3}H]TdR incorporation into CTLL-2 cells represent the mean ± SD of triplicate values of a representative experiment ($n > 3$).

~100-fold. These values are in the range of the estimations that antagonist interactions occur at affinities that are 100- to 1000-fold weaker than agonists. Taken together, these findings are quite remarkable, in that through the use of high-affinity TCRs, we are able to provide reasonable estimates for biologically relevant, but weak interactions to be occurring at millimolar affinities, a level for which there is little, if any, precedence with Ag-specific receptors.

Specificity of Ag recognition by high-affinity TCRs

Our demonstration of the increased degeneracy of Hb peptide recognition would seem on face value to contradict our previous observation that 3.L2, M4, and M15 T cells did not respond to any of the self peptides presented constitutively on APCs or the control MCC peptide (Fig. 2) (43). Accordingly, we investigated further how a T cell could have both degenerate recognition of Hb and specific recognition of other peptides. MCC and Hb bind equally well to I-E^k, and both have the same anchor residues at P1 and P9, but different P4 and P6 anchor residues. An overlay of the two peptides from the peptide-I-E^k crystal structures shows an overall similarity in the conformation of the peptide main chains of Hb and MCC; however, there are small, but significant shifts in the main chains and orientation of some side chains (P3 and P5) due to the different P4 and P6 anchor residues (Fig. 5*A*). A comparison of the amino acid sequences of the two peptides revealed prominent differences at the side chains of the exposed residues at P3 (Ala in Hb; Tyr in MCC), P5 (Asn in Hb; Lys in MCC), and P8 (Leu in Hb; Thr in MCC). Examination of our single amino acid substitutional analysis of Hb revealed that three of the MCC TCR contact residues were singly allowable, P2 (Ala), P3 (Tyr), and P8 (Thr), whereas one was not, P5 (Lys) (Fig. 3). To ascertain whether the lack of MCC response was simply due to this nonallowable P5 residue, we tested MCC single amino acid variants with either of two allowable P5 residues: the Hb residue Asn, or an Ile. Neither of these variants stimulated 3.L2, M4, or M15 (Fig. 5*B*) at concentrations up to 100 μM, demonstrating that the failure of MCC to stimulate M15 T cells was not simply the lack of allowable TCR contact residues.

To examine the effect of the other residues that differ between Hb and MCC, we generated chimeric MCC/Hb peptides in which we substituted the MHC contact residues (Fig. 6*A*). The Hb peptide with MCC residues at P4, P6, and P7 did not stimulate 3.L2, M4, or M15, revealing the influence of non-TCR contact residues on recognition by the TCR (Fig. 6*B*). Position P7 of Hb is a Gly, whereas P7 of MCC is an Ala. A single Ala substitution at P7 in Hb yielded a peptide variant that had no activity with 3.L2, weak activity with M4, and full activity with M15. Hence, position P7 had a major impact on the recognition of MCC and Hb for the high-affinity TCRs. The P7 residue has the dual potential of being involved in binding to I-E^k, and also it could be contacted by a TCR. For Hb, with a Gly at P7, the TCR could only be contacting main chain atoms, whereas in MCC or chimeric peptides containing other residues at P7, both main chain and side chain contacts would be possible. The Hb chimeric peptide having the conservative substitutions of the MCC anchor residues at P4 and P6 was also tested. This doubly substituted peptide had increased activity compared with the P7 single substitution, now being able to stimulate M4 cells weakly, and M15 cells stronger than Hb or MCC at P7 (Fig. 6*B*). (The enhanced activity relative to Hb may be the consequence of improved binding to I-E^k.) Thus, changing multiple MHC anchor residues can have dynamic effects, and not always additive. Substitution of the P7 residue alone generates a peptide that can stimulate the highest affinity TCR M15. The chimeric peptide containing MCC residues at P4 and P6 is more stimulatory than the P7 single; however, combining all three substitutions into one peptide (MCC at P4, P6, and P7) resulted in a completely nonstimulatory peptide. These observations are supported by our previous studies that showed that changes in the P6 anchor residue of Hb have minor structural effects, but significant immunological consequences (50). Thus, we suggest that the main explanation for the lack of recognition of MCC or any of the self peptides presented on APCs by the high-affinity M4 and M15 TCRs is due to small, but significant conformational changes in the peptide created by different MHC anchor residues.

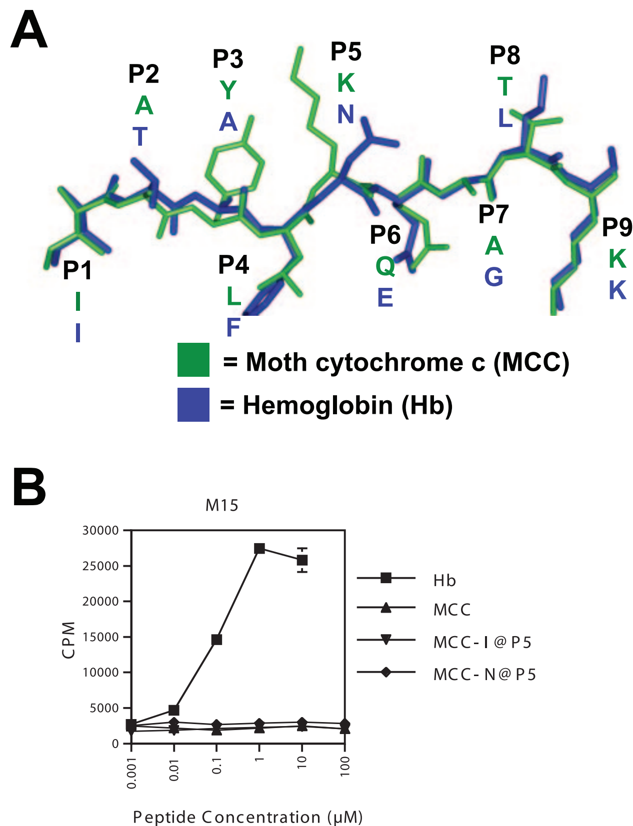


FIGURE 5. Overlay of Hb and MCC peptides and activity of MCC peptide with Hb amino acid substitution at main TCR contact residue P5. *A*, Overlay of Hb (blue) and MCC (green) peptides bound to I-E^k, showing the residues at each pocket and the overall similarity of the peptides. *B*, CD4$^+$ M15 T cell hybridomas were assayed with MCC APLs (Hb residue N or similar residue I at P5) using CH27 cells as APCs. The level of T cell stimulation was determined using a bioassay for IL-2. The cpm values of [^{3}H]TdR incorporation into CTLL-2 cells represent the mean ± SD of triplicate values. Representative results of three individual experiments are shown.

Discussion

In this study, we have used in vitro engineered high-affinity TCRs to examine TCR specificity and have made two significant findings. First, the TCR has a duality in its recognition of pMHC in that it is both degenerate and specific. Within the context of peptides that are structurally similar to the Hb peptide (i.e., single amino acid changes in solvent-accessible residues), there is significant degeneracy in the recognition. In contrast, within the context of all peptides (i.e., when the peptide MHC anchor residues and/or multiple TCR contact residues are changed), there is a high degree of specificity. The differing MHC anchor residues must produce significant movements in atoms of the peptide. These

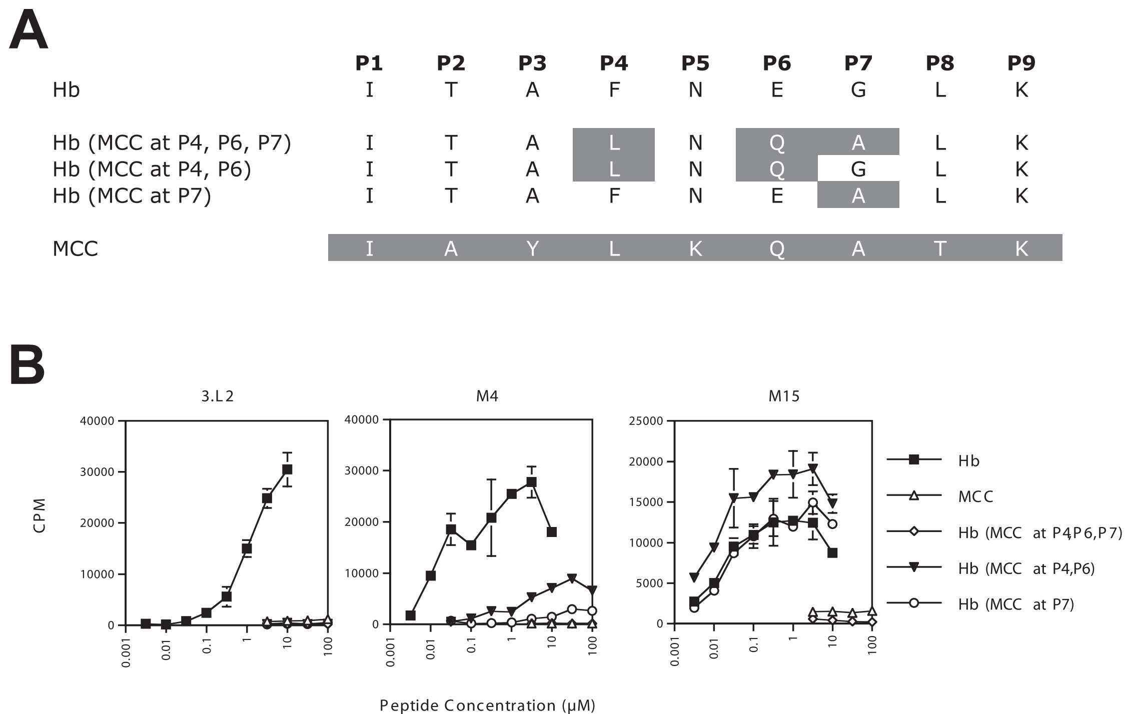


FIGURE 6. Hb, chimeric Hb/MCC, and MCC peptide alignments and activity of peptides with substitutions at nonexposed residues. *A*, Amino acid alignment of Hb, chimeric Hb/MCC, and MCC peptides (P1-P9) with the MCC amino acids highlighted in gray. *B*, T cell stimulation of 3.L2, M4, and M15 hybridomas with chimeric Hb/MCC peptides. IL-2 release is measured in a biological indicator assay (mean ± SD of triplicate values) at increasing concentrations of peptides presented by CH27 APCs. Representative results of three individual experiments are shown.

movements of some peptide atoms most likely are directly affecting contacts with the TCR, but other explanations are also possible, such as solvent or entropic effects. This provides an explanation as to why M15 TCR with its 25 nM affinity is not stimulated by any of the estimated 2000 different self pMHC complexes on an APC, which have different constellations of MHC anchor residues (51). Second, the high-affinity TCRs gave us a unique opportunity to estimate affinity values for weak, but biologically significant, TCR:pMHC interactions. Thus, like other receptors, a TCR can interact with pMHC ligands over a 1000-fold affinity range, but in the 10^{-3}–10^{-6} M range, not 10^{-6}–10^{-9} M. Clearly, interactions within the low-affinity end of the former range are impossible to measure by current methods.

For T cells to be selected in the thymus, they have to interact with endogenous positively selecting ligands. The precise nature of these ligands has not been established, but these ligands are not often going to be single amino acid variants of the agonist ligands. With the anticipatory nature of the immune system, the agonist ligands are only relevant postthymic development, and most T cells rarely, if ever, encounter an agonist ligand. Our studies with the Hb/MCC chimeric molecules revealed that changing MHC anchor residues can have both positive and negative effects on peptide recognition. Therefore, the endogenous positively selecting ligands comprise peptides that are most likely to have very different sequences from the agonist ligand, but through the combination of MHC and TCR contact residues, changes can interact productively with the TCR. Using the increased affinity window afforded by the high-affinity TCRs, we are able to estimate that these interactions can be in the 10^{-3} M range. This same phenomenon of many different chemical solutions to generate a stimulatory peptide is most likely what limits the usefulness of screening peptide libraries to identify actual agonist ligands. Peptide Ag mimics have been found and are useful as reagents, but they may or may not have any sequence similarities to the real agonist ligand (19, 30). Our findings also highlight how difficult it will be to categorize T cell recognition simply as specific or degenerate, as we and others have tried to do. If one were to use single amino acid substitutional analysis and identify many allowable side chains, the recognition may be degenerate, but it could still be highly specific when assayed with most other peptides. The sequence space represented by all possible MHC-binding peptides is more difficult to approach experimentally due to the large number of potential peptides when multiple positions are being modified.

We believe that these high-affinity TCRs are valid probes for recognition of pMHC ligands as accomplished by TCRs such as wild-type 3.L2, for several reasons. First, the high-affinity TCRs showed exquisite overall peptide specificity, as does the wild-type 3.L2. This specificity, coupled with the high affinity of the engineered TCRs, also provides valuable reagents for the tracking of specific pMHC, as has been done with several mAbs (52). Second, the fine specificity analyses indicated that although the high-affinity TCRs are more degenerate in their recognition, they have a similar hierarchy as the wild-type 3.L2 (e.g., the relative contribution of exposed residues to specificity remains the same, P8 > P5 > P3 = P2). Third, the affinity maturation pathway used to generate these TCRs yielded sequential increases in affinity that were additive and not cooperative, suggesting that there was not a reshaping of the entire TCR surface (43). Fourth, the dominant role of on-rate in the increased affinities is analogous to studies of Ab maturation pathways. Studies have documented that the bound states of the primary and matured Ab:Ag complexes are very similar, but the unbound affinity-matured Ab adopted an induced fit configuration (53, 54). Thus, it is reasonable to predict that the 3.L2 affinity-matured TCRs retained a similar bound state as 3.L2. Obviously, the structures of the wild-type 3.L2 and affinity-matured TCRs will be required to examine these issues in detail.

The results described in this study are completely consistent with the concepts of T cell activity thresholds, peptide cross-reactivity, and peptide specificity, as discussed by Garcia and colleagues (55). In their study, they propose that the observed degeneracy of a T cell occurs most often when a structurally similar peptide, with exposed residues that are identical or similar to the cognate peptide, is recognized by the TCR. Hence, the low affinity of a TCR required to elicit T cell activity (i.e., the threshold) is interpreted as degeneracy or cross-reactivity. Examination of the Hb APLs that yielded the highest degree of reactivity shows that in most cases these were conservative substitutions. The consequence is apparent degeneracy, as measured by functional activity assays, yet the T cells exhibited a high degree of overall Ag specificity to other peptides (supporting the prediction of Garcia and colleagues (55) that it is possible to retain a high degree of Ag specificity even with high-affinity TCRs).

Some aspects of the present findings are similar to our previous study, with a 2C TCR (m33α) engineered to have 1000-fold higher affinity for a foreign pMHC called SIYR/K^b, but with two notable differences (56). T cells transfected with the m33α TCR had a modest gain in Ag sensitivity (~5- to 10-fold) for SIRY/K^b, and $CD8^+$ m33α T cell hybridomas became autoreactive with self peptide/K^b complexes. The increase in Ag sensitivity could be due to the observation that the affinity of the wild-type 2C TCR for SIYR is relatively low (K_D value 30 μM). Furthermore, the autoreactivity was most likely due to stimulation by the self peptide dEV8 that is structurally related to SIYR (57). With the T cells transfected with the 3.L2 high-affinity TCRs, we did not observe such autoreactivity, perhaps because different T cells may have different propensities to become autoreactive depending on the self peptide(s). Alternatively, CD4 and CD8 cells may behave somewhat differently with an increase in affinity. Overall, through the large affinity window afforded by the high-affinity TCRs, we were able to estimate binding energies of low-affinity, biologically relevant interactions and show that T cell recognition of Ag can be both degenerate and specific.

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Disclosures

The authors have no financial conflict of interest.

References

1. Davis, M. M., J. J. Boniface, Z. Reich, D. Lyons, J. Hampl, B. Arden, and Y. Chien. 1998. Ligand recognition by $\alpha\beta$ T cell receptors. *Annu. Rev. Immunol.* 16: 523–544.
2. Krogsgaard, M., and M. M. Davis. 2005. How T cells 'see' antigen. *Nat. Immunol.* 6: 239–245.
3. Rabinowitz, J. D., C. Beeson, D. S. Lyons, M. M. Davis, and H. M. McConnell. 1996. Kinetic discrimination in T-cell activation. *Proc. Natl. Acad. Sci. USA* 93: 1401–1405.
4. McKeithan, T. W. 1995. Kinetic proofreading in T-cell receptor signal transduction. *Proc. Natl. Acad. Sci. USA* 92: 5042–5046.
5. Solinger, A. M., M. E. Ultee, E. Margoliash, and R. H. Schwartz. 1979. T-lymphocyte response to cytochrome *c*. I. Demonstration of a T-cell heteroclitic proliferative response and identification of a topographic antigenic determinant on pigeon cytochrome *c* whose immune recognition requires two complementing major histocompatibility complex-linked immune response genes. *J. Exp. Med.* 150: 830–848.
6. Hill, S. W., and E. E. Sercarz. 1975. Fine specificity of the H-2 linked immune response gene for the gallinaceous lysozymes. *Eur. J. Immunol.* 5: 317–324.
7. Zweerink, H. J., B. A. Askonas, D. Millican, S. A. Courtneidge, and J. J. Skehel. 1977. Cytotoxic T cells to type A influenza virus; viral hemagglutinin induces A-strain specificity while infected cells confer cross-reactive cytotoxicity. *Eur. J. Immunol.* 7: 630–635.
8. Braciale, T. J. 1979. Specificity of cytotoxicity T cells directed to influenza virus hemagglutinin. *J. Exp. Med.* 149: 856–869.
9. Townsend, A. R., F. M. Gotch, and J. Davey. 1985. Cytotoxic T cells recognize fragments of the influenza nucleoprotein. *Cell* 42: 457–467.
10. Gotch, F., A. McMichael, and J. Rothbard. 1988. Recognition of influenza A matrix protein by HLA-A2-restricted cytotoxic T lymphocytes: use of analogues to orientate the matrix peptide in the HLA-A2 binding site. *J. Exp. Med.* 168: 2045–2057.
11. Buus, S., A. Sette, S. M. Colon, C. Miles, and H. M. Grey. 1987. The relation between major histocompatibility complex (MHC) restriction and the capacity of Ia to bind immunogenic peptides. *Science* 235: 1353–1358.
12. Sette, A., S. Buus, S. Colon, J. A. Smith, C. Miles, and H. M. Grey. 1987. Structural characteristics of an antigen required for its interaction with Ia and recognition by T cells. *Nature* 328: 395–399.
13. Allen, P. M., G. R. Matsueda, R. J. Evans, J. B. Dunbar, Jr., G. R. Marshall, and E. R. Unanue. 1987. Identification of the T-cell and Ia contact residues of a T-cell antigenic epitope. *Nature* 327: 713–715.
14. Babbitt, B. P., P. M. Allen, G. Matsueda, E. Haber, and E. R. Unanue. 1985. Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature* 317: 359–361.
15. Kersh, G. J., and P. M. Allen. 1996. Essential flexibility in the T-cell recognition of antigen. *Nature* 380: 495–498.
16. Fujinami, R. S., M. B. Oldstone, Z. Wroblewska, M. E. Frankel, and H. Koprowski. 1983. Molecular mimicry in virus infection: cross-reaction of measles virus phosphoprotein or of herpes simplex virus protein with human intermediate filaments. *Proc. Natl. Acad. Sci. USA* 80: 2346–2350.
17. Hagerty, D. T., and P. M. Allen. 1995. Intramolecular mimicry: identification and analysis of two cross-reactive T cell epitopes within a single protein. *J. Immunol.* 155: 2993–3001.
18. Evavold, B. D., J. Sloan-Lancaster, K. J. Wilson, J. B. Rothbard, and P. M. Allen. 1995. Specific T cell recognition of minimally homologous peptides: evidence for multiple endogenous ligands. *Immunity* 2: 655–663.
19. Basu, D., S. Horvath, I. Matsumoto, D. H. Fremont, and P. M. Allen. 2000. Molecular basis for recognition of an arthritic peptide and a foreign epitope on distinct MHC molecules by a single TCR. *J. Immunol.* 164: 5788–5796.
20. Bhardwaj, V., V. Kumar, H. M. Geysen, and E. E. Sercarz. 1993. Degenerate recognition of a dissimilar antigenic peptide by myelin basic protein-reactive T cells: implications for thymic education and autoimmunity. *J. Immunol.* 151: 5000–5010.
21. Crawford, F., E. Huseby, J. White, P. Marrack, and J. W. Kappler. 2004. Mimotopes for alloreactive and conventional T cells in a peptide-MHC display library. *PLoS. Biol.* 2: E90.
22. Krogsgaard, M., N. Prado, E. J. Adams, X. L. He, D. C. Chow, D. B. Wilson, K. C. Garcia, and M. M. Davis. 2003. Evidence that structural rearrangements and/or flexibility during TCR binding can contribute to T cell activation. *Mol. Cell* 12: 1367–1378.
23. Ohteki, T., A. Hessel, M. F. Bachmann, A. Zakarian, E. Sebzda, M. S. Tsao, K. McKall-Faienza, B. Odermatt, and P. S. Ohashi. 1999. Identification of a cross-reactive self ligand in virus-mediated autoimmunity. *Eur. J. Immunol.* 29: 2886–2896.
24. Wucherpfennig, K. W., and J. L. Strominger. 1995. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. *Cell* 80: 695–705.
25. Lang, H. L., H. Jacobsen, S. Ikemizu, C. Andersson, K. Harlos, L. Madsen, P. Hjorth, L. Sondergaard, A. Svejgaard, K. Wucherpfennig, et al. 2002. A functional and structural basis for TCR cross-reactivity in multiple sclerosis. *Nat. Immunol.* 3: 940–943.
26. Loftus, D. J., Y. Chen, D. G. Covell, V. H. Engelhard, and E. Appella. 1997. Differential contact of disparate class I/peptide complexes as the basis for epitope cross-recognition by a single T cell receptor. *J. Immunol.* 158: 3651–3658.
27. Sykulev, Y., A. Brunmark, T. J. Tsomides, S. Kageyama, M. Jackson, P. A. Peterson, and H. N. Eisen. 1994. High-affinity reactions between antigen-specific T-cell receptors and peptides associated with allogeneic and syngeneic major histocompatibility complex class I proteins. *Proc. Natl. Acad. Sci. USA* 91: 11487–11491.
28. Wucherpfennig, K. W. 2004. T cell receptor cross-reactivity as a general property of T cell recognition. *Mol. Immunol.* 40: 1009–1017.
29. Reiser, J. B., C. Darnault, C. Gregoire, T. Mosser, G. Mazza, A. Kearney, P. A. van der Merwe, J. C. Fontecilla-Camps, D. Housset, and B. Malissen. 2003. CDR3 loop flexibility contributes to the degeneracy of TCR recognition. *Nat. Immunol.* 4: 241–247.
30. Judkowski, V., C. Pinilla, K. Schroder, L. Tucker, N. Sarvetnick, and D. B. Wilson. 2001. Identification of MHC class II-restricted peptide ligands, including a glutamic acid decarboxylase 65 sequence, that stimulate diabetogenic T cells from transgenic BDC2.5 nonobese diabetic mice. *J. Immunol.* 166: 908–917.
31. Udaka, K., K. H. Wiesmuller, S. Kienle, G. Jung, and P. Walden. 1996. Self-MHC-restricted peptides recognized by an alloreactive T lymphocyte clone. *J. Immunol.* 157: 670–678.
32. Daniel, C., S. Horvath, and P. M. Allen. 1998. A basis for alloreactivity: MHC helical residues broaden peptide recognition by the TCR. *Immunity* 8: 543–552.
33. Benoist, C., and D. Mathis. 2001. Autoimmunity provoked by infection: how good is the case for T cell epitope mimicry? *Nat. Immunol.* 2: 797–801.
34. Evavold, B. D., and P. M. Allen. 1991. Separation of IL-4 production from Th cell proliferation by an altered T cell receptor ligand. *Science* 252: 1308–1310.
35. Kersh, G. J., D. L. Donermeyer, K. E. Frederick, J. M. White, B. L. Hsu, and P. M. Allen. 1998. TCR transgenic mice in which usage of transgenic α- and β-chains is highly dependent on the level of selecting ligand. *J. Immunol.* 161: 585–593.

36. Williams, C. B., D. L. Engle, G. J. Kersh, J. Michael White, and P. M. Allen. 1999. A kinetic threshold between negative and positive selection based on the longevity of the T cell receptor-ligand complex. *J. Exp. Med.* 189: 1531–1544.
37. Kao, H., and P. M. Allen. 2005. An antagonist peptide mediates positive selection and CD4 lineage commitment of MHC class II-restricted T cells in the absence of CD4. *J. Exp. Med.* 201: 149–158.
38. Grakoui, A., S. K. Bromley, C. Sumen, M. M. Davis, A. S. Shaw, P. M. Allen, and M. L. Dustin. 1999. The immunological synapse: a molecular machine controlling T cell activation. *Science* 285: 221–227.
39. Li, Y., Y. Huang, J. Lue, J. A. Quandt, R. Martin, and R. A. Mariuzza. 2005. Structure of a human autoimmune TCR bound to a myelin basic protein self-peptide and a multiple sclerosis-associated MHC class II molecule. *EMBO J.* 24: 2968–2979.
40. Shusta, E. V., P. D. Holler, M. C. Kieke, D. M. Kranz, and K. D. Wittrup. 2000. Directed evolution of a stable scaffold for T-cell receptor engineering. *Nat. Biotechnol.* 18: 754–759.
41. Kieke, M. C., E. V. Shusta, E. T. Boder, L. Teyton, K. D. Wittrup, and D. M. Kranz. 1999. Selection of functional T cell receptor mutants from a yeast surface-display library. *Proc. Natl. Acad. Sci. USA* 96: 5651–5656.
42. Holler, P. D., P. O. Holman, E. V. Shusta, S. O'Herrin, K. D. Wittrup, and D. M. Kranz. 2000. In vitro evolution of a T cell receptor with high-affinity for peptide/MHC. *Proc. Natl. Acad. Sci. USA* 97: 5387–5392.
43. Weber, K. S., D. L. Donermeyer, P. M. Allen, and D. M. Kranz. 2005. Class II-restricted T cell receptor engineered in vitro for higher affinity retains peptide specificity and function. *Proc. Natl. Acad. Sci. USA* 102: 19033–19038.
44. Vidal, K., C. Daniel, M. Hill, D. R. Littman, and P. M. Allen. 1999. Differential requirements for CD4 in TCR-ligand interactions. *J. Immunol.* 163: 4811–4818.
45. Reay, P. A., R. M. Kantor, and M. M. Davis. 1994. Use of global amino acid replacements to define the requirements for MHC binding and T cell recognition of moth cytochrome *c* (93-103). *J. Immunol.* 152: 3946–3957.
46. Evavold, B. D., S. G. Williams, B. L. Hsu, S. Buus, and P. M. Allen. 1992. Complete dissection of the Hb(64-76) determinant using T helper 1, T helper 2 clones, and T cell hybridomas. *J. Immunol.* 148: 347–353.
47. Fremont, D. H., W. A. Hendrickson, P. Marrack, and J. Kappler. 1996. Structures of an MHC class II molecule with covalently bound single peptides. *Science* 272: 1001–1004.
48. Fremont, D. H., S. Dai, H. Chiang, F. Crawford, P. Marrack, and J. Kappler. 2002. Structural basis of cytochrome *c* presentation by IE^k. *J. Exp. Med.* 195: 1043–1052.
49. Kersh, G. J., and P. M. Allen. 1996. Structural basis for T cell recognition of altered peptide ligands: a single T cell receptor can productively recognize a large continuum of related ligands. *J. Exp. Med.* 184: 1259–1268.
50. Kersh, G. J., M. J. Miley, C. A. Nelson, A. Grakoui, S. Horvath, D. L. Donermeyer, J. Kappler, P. M. Allen, and D. H. Fremont. 2001. Structural and functional consequences of altering a peptide MHC anchor residue. *J. Immunol.* 166: 3345–3354.
51. Suri, A., I. Vidavsky, K. van der Drift, O. Kanagawa, M. L. Gross, and E. R. Unanue. 2002. In APCs, the autologous peptides selected by the diabetogenic I-Ag7 molecule are unique and determined by the amino acid changes in the P9 pocket. *J. Immunol.* 168: 1235–1243.
52. Germain, R. N., and M. K. Jenkins. 2004. In vivo antigen presentation. *Curr. Opin. Immunol.* 16: 120–125.
53. Wedemayer, G. J., P. A. Patten, L. H. Wang, P. G. Schultz, and R. C. Stevens. 1997. Structural insights into the evolution of an antibody combining site. *Science* 276: 1665–1669.
54. Yin, J., E. C. Mundorff, P. L. Yang, K. U. Wendt, D. Hanway, R. C. Stevens, and P. G. Schultz. 2001. A comparative analysis of the immunological evolution of antibody 28B4. *Biochemistry* 40: 10764–10773.
55. Maynard, J., K. Petersson, D. H. Wilson, E. J. Adams, S. E. Blondelle, M. J. Boulanger, D. B. Wilson, and K. C. Garcia. 2005. Structure of an autoimmune T cell receptor complexed with class II peptide-MHC: insights into MHC bias and antigen specificity. *Immunity* 22: 81–92.
56. Holler, P. D., L. K. Chlewicki, and D. M. Kranz. 2003. TCRs with high-affinity for foreign pMHC show self-reactivity. *Nat. Immunol.* 4: 55–62.
57. Degano, M., K. C. Garcia, V. Apostolopoulos, M. G. Rudolph, L. Teyton, and I. A. Wilson. 2000. A functional hot spot for antigen recognition in a superagonist TCR/MHC complex. *Immunity* 12: 251–261.