nature immunology

Interface-disrupting amino acids establish specificity between T cell receptors and complexes of major histocompatibility complex and peptide

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T cell receptors (TCRs) bind complexes of cognate major histocompatibility complex (MHC) and peptide at relatively low affinities (1–200 μM). Nevertheless, TCR-MHC-peptide interactions are usually specific for the peptide and the allele encoding the MHC. Here we show that to escape thymocyte negative selection, TCRs must interact with many of the side chains of MHC-peptide complexes as 'hot spots' for TCR binding. Moreover, even when the 'parental' side chain did not contribute binding affinity, some MHC-peptide residues contributed to TCR specificity, as amino acid substitutions substantially reduced binding affinity. The presence of such 'interface-disruptive' side chains helps to explain how TCRs generate specificity at low-affinity interfaces and why TCRs often 'accommodate' a subset of amino acids at a given MHC-peptide position.

During T cell development, each thymocyte generates through recombination the genes required for expression of its T cell receptor (TCR) for major histocompatibility complex (MHC) protein–peptide ligands[1]. Depending on the quality of interaction between the displayed TCR and self peptides bound to host MHC proteins, the thymocyte receives a signal to live or die. Many experiments have demonstrated that thymocyte selection is driven by the affinity of a TCR for MHC-peptide. Thymocytes bearing TCRs with no appreciable affinity for host MHC bound to self peptides die, whereas thymocytes bearing TCRs with high affinity for host MHC bound to self peptides die by negative selection. Only thymocytes expressing TCRs with a relatively moderate affinity and/or avidity for self peptides bound to host MHC proteins undergo positive selection and are exported to the periphery[2–7].

However, the reason why TCRs on mature T cells are usually specific for particular classes and alleles of MHC and for a small set of foreign peptides is not known. Although about 2–5% of TCRs on mature T cells in wild-type mice can react with more than one allele of MHC and although TCRs often react with more than one peptide, on the whole, the cross-reactivity of TCRs on mature T cells is relatively limited[8,9]. Such limited cross-reactivity is not found in the preselection thymocyte repertoire, however. Several experiments have shown, for example, that in the absence of positive and negative selection, as many as 20% of thymocytes can react with self peptides bound to MHC[10–13]. Although the peptide specificity of those preselection TCRs is unknown, such a high frequency of self-reactivity indicates that the random repertoire of thymocyte TCRs is biased toward recognition of the MHC and its associated peptides and that somehow selection in the thymus eliminates most of the thymocytes bearing MHC-reactive TCRs. Initially it was suggested that positive selection requires interaction between the TCRs on developing thymocytes with peptide- and MHC allele–specific determinants in the thymus[14–18]. The specificity of the mature T cell would therefore be selected by requirements dictated by positive selection.

Experiments have suggested, however, that the phenomenon is controlled by negative rather than positive selection[19]. Those studies involved T cells in mice that express MHC class II bound to only one peptide[20]. Negative selection of $CD4^+$ T cells is limited in such mice because they have only one potentially negatively selecting ligand rather than the many available in wild-type mice, in which MHC class II is engaged by thousands of peptides[20–23]. Some $CD4^+$ T cells from the mice that express MHC class II bound to only one peptide are very cross-reactive, responding to MHC class II bound to many different peptides, to many different alleles of MHC class II and even to MHC class I proteins[19]. Those results led to the suggestion that the family of TCRs contains some that are 'promiscuous' in their interactions with MHC-peptide. Usually thymocytes bearing those TCRs are eliminated, because in wild-type mice, the thymocytes are likely to encounter a combination of self MHC–self peptide with which they interact and are thereby killed by negative selection. Thymocytes bearing such cross-reactive TCRs are less likely to encounter a negatively selecting self MHC–self peptide combination in mice that express MHC class II bound to only one peptide.

To elucidate the cross-reactivity of TCRs, here we have studied in detail the reactions of two specific and three cross-reactive TCRs with a known MHC-peptide ligand and specific amino acid substitution

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Received 13 June; accepted 20 September; published online 15 October 2006; doi:10.1038/ni1401

thereof. The five TCRs had similar kinetics and affinity of binding to the MHC-peptide combination of I-A^b plus the 'model' peptide '3K' ('I-A^b + 3K') against which they were raised[19], and thus affinity alone cannot account for the different properties of the two types of TCRs (specific and cross-reactive). Three other, not necessarily mutually exclusive, explanations might apply. First, the cross-reactive TCRs might react productively with many more amino acid side chains of the MHC-peptide complex than specific TCRs do, but do so more weakly with each individual side chain. Hence, a change in a recognized MHC-peptide side chain would have less effect on the overall affinity of a cross-reactive TCR than on that of a specific TCR. Second, the cross-reactive TCRs might recognize fewer MHC-peptide side chains than the specific TCRs do but might gain more energy from reaction with each recognized side chain. Thus, many of the MHC-peptide side chains could be altered without affecting the affinity of cross-reactive TCRs while substantially changing the affinity of specific TCRs. Third, the cross-reactive TCRs might gain more of their binding energy from interaction with some feature of the MHC-peptide that is not specific to the side chain, such as its backbone carbonyl, amino groups and α- and β-carbons.

Here, comparison of the biophysics of the reactions between the three cross-reactive and two specific TCRs and variants of I-A^b + 3K showed that the size and location of their 'footprints' on MHC-peptide complexes were similar for both specific and cross-reactive TCRs. However, the specific TCRs reacted with high binding energy with more MHC-peptide side chains than did the cross-reactive TCRs. Additionally, we found that the side chains of the MHC-peptide governed the specificity of their reaction with TCRs in two ways. First, as expected, some side chains of the MHC-peptide were recognized specifically by TCRs. Second, and unexpectedly, many side chains at positions surrounding the high-affinity binding site of the MHC-peptide complex that were not recognized by the TCR were nevertheless at positions that contributed to the specificity of the TCR-MHC-peptide interaction. Those potentially 'disruptive' side-chain residues tended to be in a circle around the amino acids whose side chains were recognized with high affinity. The side chains that disrupted the TCR-MHC-peptide interface varied in their chemical character and were not necessarily large bulky side chains. Thus, our data presented here, which support the third hypothesis described above, provide additional insight into the understanding of TCR interactions with their ligands, indicating the existence of potentially 'disruptive' residues at particular sites in the interaction surface that contribute specificity to TCR-MHC-peptide interactions.

RESULTS

Cross-reactive TCRs

A collection of T cells reactive to I-A^b + 3K has been used to study the effects of negative selection on TCR repertoires. To do that, such cells were isolated from normal C57BL/6 mice and from I-A^b 'single-peptide' mice in which negative selection was limited. Experiments with those T cells showed that the TCRs from the normal C57BL/6 mice were very specific in their recognition of I-A^b + 3K[19]. In contrast, some of the I-A^b + 3K–reactive T cells from mice with limited negative selection were very cross-reactive[19]. In this study, we have categorized a TCR as 'peptide cross-reactive' if it responded to many more amino acid substitutions of the peptide than did TCRs from C57BL/6 mice[19]. Likewise, for the TCR to be defined as 'MHC cross-reactive', it had to respond to many alleles of MHC, unlike most TCRs from C57BL/6 mice.

To follow up on those observations, we measured the affinity with which the TCRs expressed on the T cells bound I-A^b + 3K and variants thereof to determine whether the specific TCRs differed detectably from the cross-reactive TCRs in their 'footprint' on I-A^b + 3K or in the number of amino acid side chains of I-A^b + 3K that contributed substantial energy to the binding reactions. Two of the TCRs derived from mice with limited negative selection, YAe62.8 and 75-1, were peptide cross-reactive and MHC cross-reactive, whereas the third, 2W20.4, was peptide specific and MHC cross-reactive. In contrast, the two TCRs from C57BL/6 mice, B3K506 and B3K508 were both peptide specific and MHC specific (**Supplementary Table 1** online). We used two different methods to make those measurements: surface plasmon resonance and a cell-staining reaction.

Biacore measurement of kinetics and affinities

We prepared soluble versions of the five TCRs and immobilized them on surface plasmon resonance biosensor flow cells[19]. We also produced soluble preparations of I-A^b + 3K and of I-A^b bound to variants of 3K in which one of the five solvent-accessible amino acids of the peptide was replaced with alanine or another amino acid. We determined the sequence and configuration of the wild-type 3K peptide when bound to I-A^b (**Fig. 1**) and chose certain 3K peptide substitutions for analysis (**Table 1**). We passed the soluble preparations of the I-A^b + 3K variants with amino acid substitutions over the TCR-bound biosensor chips and measured the 'on rates' and 'off rates' and equilibrium dissociation rates of the combinations (**Table 1**). The strength of the TCR-MHC-peptide interaction (free energy of binding (ΔG)) is related to the natural log of affinity ($\Delta G = -RT\ln(K_d^{-1})$, where R is the gas constant 1.987 cal/mol T is the temperature in Kelvin, and K_d is the dissociation constant). Thus, a change in the free energy of binding ($\Delta\Delta G$) of 1.5 kcal/mol would change a highly stimulatory interaction of TCR-MHC-peptide with a K_d of 10 μM into a barely detectable interaction with a K_d of 125 μM. Side chains contributing 1.5 kcal/mol or more are often called 'hot spots' of binding and are generally found in the center of the binding site[24,25]. Because our studies demonstrated the change in K_d caused by each substitution of I-A^b + 3K amino acids from wild-type to variant, we were able to calculate the change in free energy of binding produced by each substitution (**Table 1**).

Measurement of affinity with a staining assay

We sought to extend those observations to studies of other substitutions in the peptide and I-A^b residues that might engage the TCRs. However, doing such experiments using surface plasmon resonance would require high-quality soluble versions of each substitution, which would be prohibitively expensive and time consuming. We

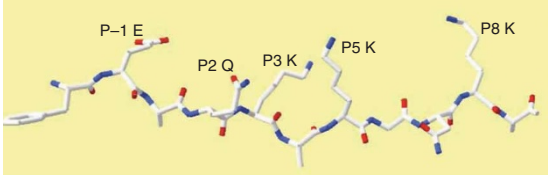

Figure 1 Side chains of the MHC-bound peptide can contribute specificity to recognition by TCRs even if the 'parental' side chain does not contribute binding energy to the reaction. This structure of the 3K peptide shows the five solvent-accessible side chains[29]: P–1 E, glutamic acid at position '–1'; P2 Q, glutamine at position 2; P3 K, lysine at position 3; P5 K, lysine at position 5; P8 K, lysine at position 8. The complete sequence of the 3K peptide is FEAQKAKANKA.

Table 1 Affinities and kinetics of the binding of 3K-specific TCRs for wild-type I-A^b + 3K and peptide mutants by surface plasmon resonance

TCR	I-A^b + 3K[a]	K_d (μM)	K_a (1/M)	k_a (1/s × M)	k_d (1/s)	ΔG (kcal/mol)	$\Delta\Delta G$ (kcal/mol)
B3K506	WT	7	136,986	102,997	0.8	–7.0	0.0
B3K506	P–1 A	26	38,462	100,288	2.6	–6.3	0.7
B3K506	P–1 L	122	8,200	ND	ND	–5.3	1.7
B3K506	P–1 K	101	9,900	61,182	6.2	–5.4	1.6
B3K506	P2 A	278	3,597	ND	ND	–4.8	2.2
B3K506	P3 A	>550	<1,818	ND	ND	>–4.4	>2.6
B3K506	P5 A	>550	<1,818	ND	ND	>–4.4	>2.6
B3K506	P5 R	11	95,100	79,575	0.8	–6.8	0.2
B3K506	P8 A	92	10,870	39,022	3.6	–5.5	1.5
B3K506	P8 G	69	14,400	67,872	4.7	–5.7	1.3
B3K506	P8 L	256	3,906	ND	ND	–4.9	2.1
B3K506	P8 Q	114	8,772	ND	ND	–5.4	1.6
B3K508	WT	29	34,130	11,766	0.3	–6.2	0.0
B3K508	P–1 A	>550	<1,818	ND	ND	>–4.4	>1.7
B3K508	P–1 L	>550	<1,818	ND	ND	>–4.4	>1.7
B3K508	P–1 K	>550	<1,818	ND	ND	>–4.4	>1.7
B3K508	P2 A	175	5,714	ND	ND	–5.1	1.1
B3K508	P3 A	>550	<1,818	ND	ND	>–4.4	>1.7
B3K508	P5 A	>550	<1,818	ND	ND	>–4.4	>1.7
B3K508	P5 R	93	10,700	11,610	1.1	–5.5	0.7
B3K508	P8 A	>550	<1,818	ND	ND	>–4.4	>1.7
YAe62.8	WT	8	119,048	83,413	0.7	–6.9	0.0
YAe62.8	P–1 A	9	113,636	75,174	0.7	–6.9	0.0
YAe62.8	P–1 L	15	66,400	104,027	1.6	–6.6	0.3
YAe62.8	P–1 K	22	45,500	95,891	2	–6.4	0.6
YAe62.8	P2 A	56	17,857	ND	ND	–6.4	1.1
YAe62.8	P3 A	62	16,129	ND	ND	–5.7	1.2
YAe62.8	P5 A	>550	<1,818	ND	ND	>–4.4	>2.5
YAe62.8	P5 R	19	53,300	92,564	1.7	–6.4	0.5
YAe62.8	P8 A	7	151,515	112,500	0.7	7.1	–0.1
YAe62.8	P8 G	6	170,500	73,400	0.4	–7.1	–0.2
YAe62.8	P8 L	54	18,519	108,272	5.8	–5.8	1.1
YAe62.8	P8 Q	39	25,641	40,321	1.6	–6.0	0.9
75-1	WT	8	128,205	104,829	0.8	–7.0	0.0
75-1	P–1 A	1.1	128,205	239,545	0.3	–8.1	–1.2
75-1	P2 A	21	47,619	15,810	0.3	–6.4	0.6
75-1	P3 A	50	20,000	ND	ND	–5.9	1.1
75-1	P5 A	>650	<1,538	ND	ND	>–4.3	>2.6
75-1	P8 A	>650	<1,538	ND	ND	>–4.3	>2.6
2W20.4	WT	14	73,529	28,529	0.4	–6.6	0.0
2W20.4	P–1 A	0.5	2,000,000	264,250	0.1	–8.6	–2.0
2W20.4	P2 A	18	55,556	87,222	1.6	–6.5	0.2
2W20.4	P3 A	>650	<1,538	ND	ND	>–4.3	> 2.3
2W20.4	P5 A	>650	<1,538	ND	ND	>–4.3	> 2.3
2W20.4	P8 A	18	55,556	123,750	2.2	–6.5	0.2

[a]Includes position (P) and amino acid substitution.

therefore used fluorescence-labeled soluble TCR multimers to stain cells expressing membrane-bound wild-type I-A^b + 3K or I-A^b + 3K with amino acid substitutions at potential TCR contacts of the peptide or MHC[19,26]. The validity of that method was based on our finding of a direct relationship between the equilibrium affinity measured by surface plasmon resonance and the mean fluorescence intensity (MFI) of TCR multimer staining (**Fig. 2**). By comparing the MFI of TCR multimer staining of membrane-bound I-A^b + 3K variants to the affinity measured by surface plasmon resonance, we created a standard curve relating affinity to TCR multimer staining (**Fig. 2e**). Affinities measured by TCR multimer staining had an average of only 10% variance from the equilibrium affinity measured by surface plasmon resonance using this standard curve and less than that in the range at which the values were most reliable, between 5 μM and 250 μM (**Supplementary Table 2** online).

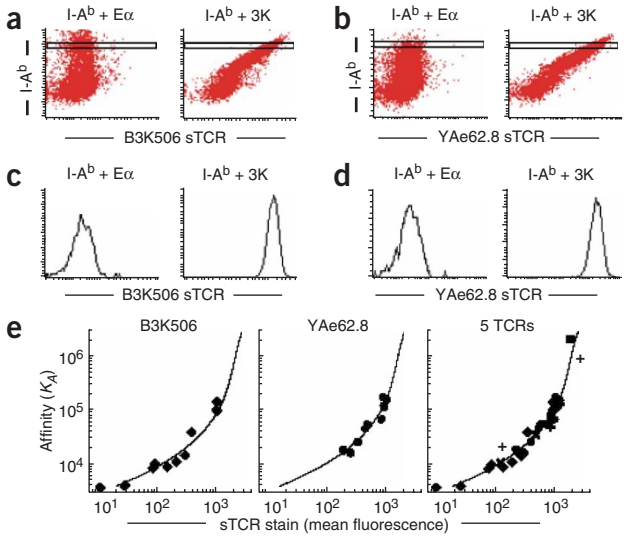

Figure 2 Construction of a standard curve of the binding of TCRs to wild-type I-A^b + 3K and mutants of I-A^b + 3K displayed on insect cells relative to the known affinities of each interaction. (**a**,**b**) Flow cytometry of insect cells infected with viruses expressing I-A^b + Eα (irrelevant peptide; negative control) or I-A^b + 3K and stained with antibody to I-A^b and multimers of the B3K506 TCR (**a**) or the YAe62.8 TCR (**b**). Cells with intermediate to high I-A^b staining were obtained (narrow rectangular outlined areas). (**c**,**d**) Staining of the cells in **a**,**b** with the B3K506 and YAe62.8 TCRs. (**e**) Mean fluorescence of the TCR staining in **c**,**d** and others obtained for the binding of soluble TCRs to I-A^b + 3K variants, plotted against the known affinities for each pair (**Table 1**). K_A values used were such that an increase in binding strength corresponds to a large value. Plots of affinity versus TCR multimer staining are for B3K506, YAe62.8 or all five TCRs. sTCR, soluble TCR.

Such assays have usually been done in reverse, using cells expressing TCRs to measure reactions with soluble MHC-peptide oligomers[27,28]. However, the assays seem equally valid when done as described here, as demonstrated by the quality of the standard curve (**Fig. 2e**). The results do not depend on the TCRs and MHC-peptide combinations used, as the relationship between equilibrium affinities measured by surface plasmon resonance and TCR multimer staining also held true for a human TCR recognizing peptides bound to HLA-DR52c and a mouse TCR recognizing peptides bound to H-2L^d (unpublished observations). Using TCR multimer staining and this standard curve, we generated $\Delta\Delta G$ data for the interaction of the five TCRs with each of the five peptide and ten MHC amino acids that might make contact with the TCR, in which each residue being studied was replaced with alanine or with one of four other amino acids (**Fig. 3**).

In each experiment, we measured the MFI of TCR multimer staining for more than 75 different substitutions of I-A^b + 3K. Cell surface display of I-A^b + 3K substitutions by recombinant baculovirus infection of insect cells led to slight variability of expression of each I-A^b + 3K variant, due to the timing of cellular infection[26]. Thus, when assessing TCR multimer staining, we selected an expression amount common to all the I-A^b + 3K variants, which was the highest expression that all I-A^b + 3K substitutions reached. That allowed measurement of TCR multimer staining with the greatest sensitivity and gave the maximum opportunity for measuring binding of even those TCRs with low affinity for the I-A^b + 3K substitution being studied. The relationship between equilibrium affinity and TCR multimer staining was not affected when we used an I-A^b + 3K variant with lower expression for analysis (data not shown). However, when we used lower cell surface expression of I-A^b + 3K to determine TCR affinity by staining with TCR multimers, the low-affinity ligand interactions, not unexpectedly, became indistinguishable from background (data not shown).

It was possible that some of the I-A^b + 3K substitutions used in these experiments inhibited folding of the MHC-peptide complex, caused the peptide to lose its ability to bind I-A^b or caused a frameshift in the register with which the peptide bound MHC. We do not believe any of the I-A^b + 3K variants we studied had those problems, however, for several reasons. I-A^b is not expressed on the cell surface of insect cells if it is denatured or in the absence of a covalently bound peptide (data not shown). Moreover, the four pockets of I-A^b with which it binds peptide are shallow and neutrally charged[29]. Because the 3K peptide has three large, negatively charged lysine residues at solvent-accessible sites, it is very unlikely that a peptide-binding register frameshift could occur, as such a shift would force one or more of the lysine residues into the shallow peptide binding pockets of I-A^b, which is an unlikely scenario.

Cross-reactive TCRs use fewer side chains of I-A^b + 3K

We used the I-A^b + 3K amino acid substitutions to answer the following two questions: which amino acid side chains contribute binding energy, and which residues of the interface contribute specificity? We used substitution with alanine to map the binding contribution of each side chain and substitution with the other amino acids to map the specificity of the TCR reactions. There are some caveats to the alanine-scanning method of mapping binding contributions of amino acid side chains (described in the Discussion section). However, the simplest interpretation of the alanine-substitution data is that they demonstrate the energy contributed to the reaction with TCRs by the side chain of that amino acid, past the β-carbon of the residue being studied[25].

For the specific TCRs B3K506 and B3K508, four of the five alanine substitutions resulted in a $\Delta\Delta G$ of 1.5 kcal/mol or more. Conversely, for the cross-reactive TCRs YAe62.8, 75-1 and 2W20.4, only one or two alanine substitutions had an effect of that magnitude (**Table 1** and **Fig. 3b–f**). Likewise, for the MHC variants with substitutions to alanine, of the eight positions that were not alanine residues in the wild-type protein and that could therefore be analyzed in this way, three or four contributed 1.5 kcal/mol or more to the binding of the specific TCRs. In contrast, only one or two of the I-A^b variants with substitutions to alanine had the same large effect on the binding energy of the cross-reactive TCRs (**Fig. 3b–e**). However, when we analyzed the data for side chains that contributed at least moderate amounts of binding affinity ($\Delta\Delta G$ of 0.8 kcal/mol or more), all five TCRs recognized approximately the same number of amino acid side chains. Our data (**Table 2**) show that in total the two specific TCRs used seven or eight side chains of I-A^b + 3K as hot spots for binding, as defined by a $\Delta\Delta G$ of 1.5 kcal/mol or more, whereas the cross-reactive TCRs used only between two and four side chains of I-A^b + 3K residues to contribute high-affinity binding.

We categorized those results according to the effects of the substitution of each of the I-A^b + 3K amino acids to alanine on the free energy of binding of the TCRs. For simplicity, we assigned the effects of substitutions of I-A^b + 3K amino acids to one of four 'color-coded' categories (**Fig. 4**), with the colors identifying changes in binding energies that would convert an interaction with a K_d of 10 μM (the average for many TCR-MHC-peptide interactions) into interactions with K_d values of 17 μM, 39 μM or 125 μM ($\Delta\Delta G = 0.3$–0.8 kcal/mol,

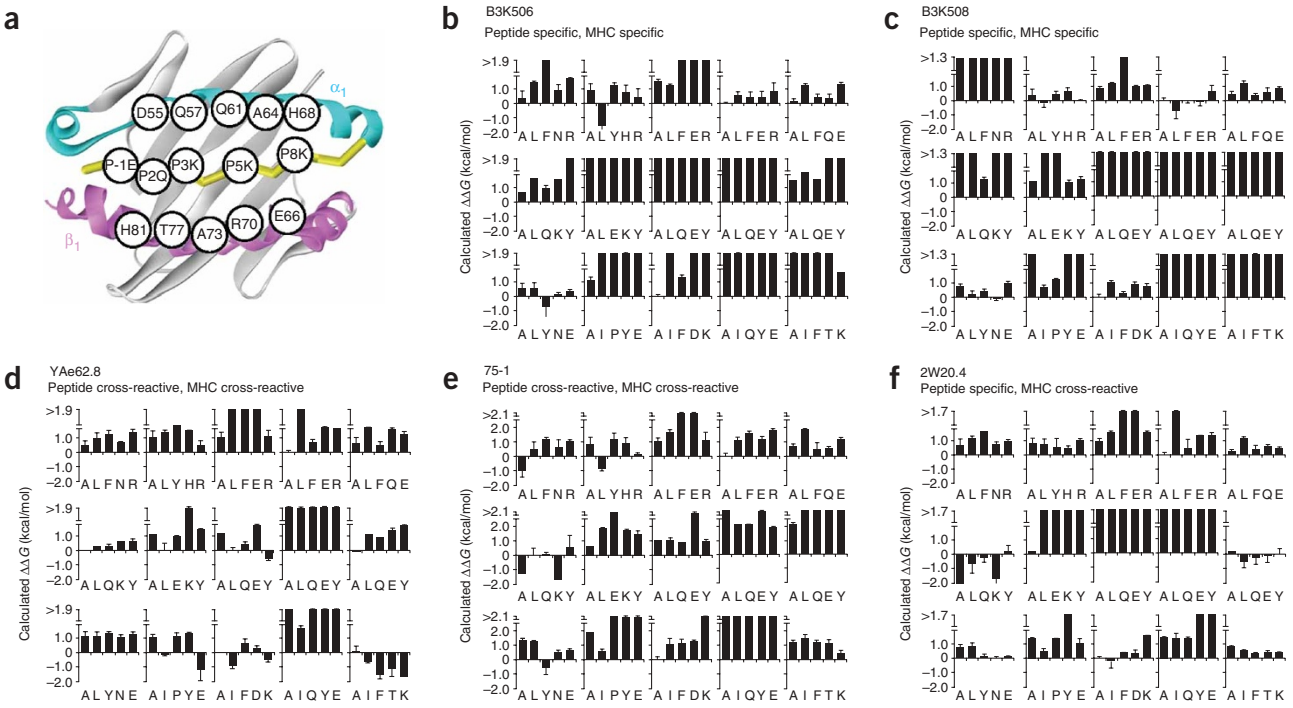

Figure 3 The $\Delta\Delta G$ for TCRs binding to I-A^b + 3K with amino acid substitutions at each of the five potential TCR contact residues of the I-A^b α-chain, peptide and I-A^b β-chain. (**a**) The 15 amino acid residues of I-A^b + 3K substituted here[29]. (**b**–**f**) $\Delta\Delta G$ data for the B3K506 TCR (**b**), B3K508 TCR (**c**), YAe62.8 TCR (**d**), 75-1 TCR (**e**) and 2W20.4 TCR (**f**). Top row, I-A^b α-chain residues (left to right, α55, α57, α61, α64 and α68); middle row, peptide (left to right, positions '–1', 2, 3, 5 and 8); bottom row, I-A^b β-chain (left to right, β81, β77, β73, β70 and β66). Letters under bars indicate amino acids after substitution. The $\Delta\Delta G$ values are those measured by surface plasmon resonance (**Table 1**) or, if not directly measured, calculated from TCR multimer staining using the standard curve (**Fig. 2**). Each substitution mutant of I-A^b + 3K was stained at least three independent times with the same settings (error bars, s.d. of the independent measurements).

0.8–1.5 kcal/mol, or 1.5 kcal/mol or more, respectively). When 'mapped' onto the structure of I-A^b + 3K, the side chains contributing most of the free energy of binding to each TCR comprised a relatively contiguous patch (**Fig. 4a–e**).

Those data collectively indicated that the cross-reactive TCRs bound fewer side chains of the MHC-peptide complex with high affinity than did the specific TCRs. However, the size and location of the interface with I-A^b + 3K of the specific and cross-reactive TCRs was similar. Thus, the cross-reactive TCRs either generated a very large amount of binding energy from just a few side chains or generated more binding energy from main-chain positions of the MHC-peptide complex than did MHC-peptide–specific TCRs. In either case, the cross-reactive TCRs required relatively fewer side chains of the MHC-peptide interface to generate sufficient binding energy for the activation of T cells, which probably contributed to their relative cross-reactivity.

Identification of inhibitory side chains of the peptide and MHC

In a few cases, the alanine substitution increased rather than decreased the strength of binding between the TCR and the MHC-peptide complex. For example, the 75-1 and 2W20.4 TCRs had a very large increase in the strength of binding to I-A^b + 3K ($\Delta\Delta G$ values of –1.2 kcal/mol and –2.0 kcal/mol, respectively; **Table 1**) when position '–1' of the peptide was replaced with alanine, indicating that the glutamic acid at position '–1' of the 'parental' side chain actually inhibited the binding of those TCRs. Likewise, analysis of the effects of alanine substitution in I-A^b showed that the replacement of aspartic acid with alanine at position 55 of I-A^b increased the energy of

Table 2 MHC plus peptide side chains contributing substantial or moderate binding energy

TCR	Peptide	MHC	Peptide (≥1.5 kcal/mol)	MHC (≥1.5 kcal/mol)	Total (≥1.5 kcal/mol)	Peptide (≥0.8 kcal/mol)	MHC (≥0.8 kcal/mol)	Total (≥0.8 kcal/mol)
B3K506	Specific	Specific	4	3	7	4	5	9
B3K508	Specific	Specific	4	4[a]	8	5	4	9
YAe62.8	Cross-reactive	Cross-reactive	1	1	2	3	5	8
75-1	Cross-reactive	Cross-reactive	2	2	4	3	5	8
2W20.4	Specific	Cross-reactive	2	0	2	2	6	8

Binding energies were calculated from surface plasmon resonance (peptide mutants) or TCR multimer staining (I-A^b mutants) by comparison of the binding of TCR to wild-type I-A^b + 3K versus I-A^b + 3K with alanine substitution.
[a]Four side chains contributed more than 1.3 kcal/mol of binding energy, the limit of detection for this TCR by multimer staining.

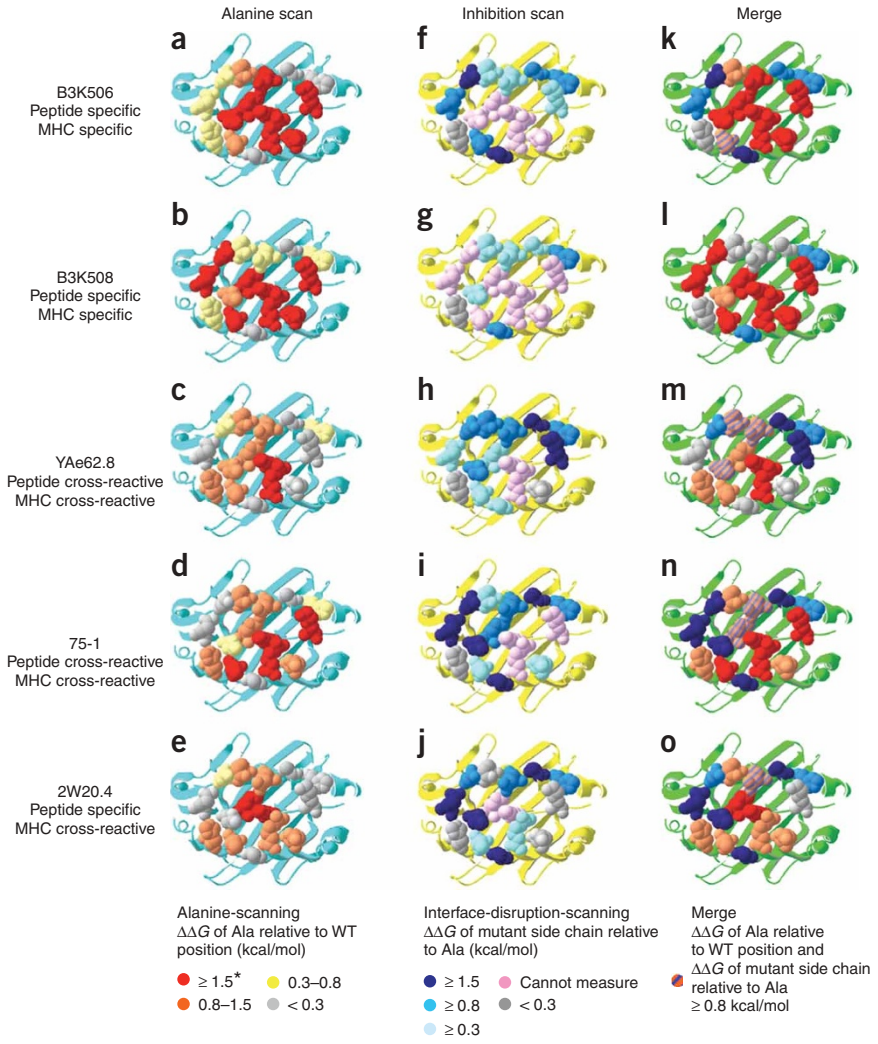

Figure 4 'Footprint' analyses of the side chains of I-A^b + 3K that contribute binding affinity or contribute specificity through the ability to disrupt the binding of TCRs. (**a**–**e**) Contributions of amino acids of the wild-type (WT) side chain to binding affinity, determined by the effects of alanine substitution. (**f**–**j**) Analysis of whether substitutions at an amino acid position can disrupt TCR binding, compared with alanine at that position. (**k**–**o**) Merge of the alanine-scanning data (**a**–**e**) and interface-disruption-scanning data (**f**–**j**). *, four side chains of I-A^b contributed more than 1.3 kcal/mol of binding energy for B3K508, the limit of detection for this TCR by multimer staining. Colors of the amino acids indicate their contribution to binding[29] (keys, bottom).

binding of the 75-1 TCR (**Fig. 3**). Those effects may be examples of what has been described in the past as 'heteroclitic reactions', in which T cells react better with altered MHC or peptides than they do with the wild-type molecules. Often heteroclitic reactions have turned out to be the result of higher affinity binding of the altered peptide by MHC and therefore the availability of more ligand to stimulate T cells. In some cases, however, heteroclitic reactions may be due, as we noted, to higher affinity of the variant MHC-peptide complex for the TCR being studied[30,31].

MHC and peptide 'interface-disrupting' positions

Although some side chains contributed substantial binding energy while others were actually inhibitory (discussed above) to the reaction of I-A^b + 3K with the TCRs, substitution of alanine for amino acids at other positions sometimes had no effect on the energy with which TCRs bound the mutant I-A^b + 3K. For example, the substitution of alanine for histidine at position 68 of I-A^b α-chain had little effect on the binding energy of any of the TCRs, the substitution of alanine for glutamine at position 2 of the 3K peptide had little or no effect on recognition by the 75-1 and 2W20.4 TCRs, and the substitution of alanine for lysine at position 8 of 3k had little effect on binding by the YAe62.8 TCR (**Figs. 1b** and **3**). Such results suggested that those residues were irrelevant for TCR-MHC-peptide binding. However, it has been shown that some substitutions at those residues abolish the activation of T cells bearing the relevant TCRs. Because activation of T cells is a complex signaling event and some nonactivating ligands can have relatively high affinities, it was unclear what caused that discrepancy[32–34].

To resolve that issue, we assessed the effect of the replacement of I-A^b + 3K amino acids with residues other than alanine (**Table 1** and **Fig. 3b–f**). To 'map' the specificity of each residue in an unbiased way, we used four chemically dissimilar side chains at each position. Many of the amino acids introduced in these substitutions are present in naturally occurring MHC molecules. In all cases in which an alanine substitution was not tolerated, a substitution in that position to other amino acids not related to the 'parental' amino acid was also generally not tolerated (such as the effect of changes at positions 2, 3, 5 and 8 and the switch to arginine at position 70 of I-A^b β-chain and to glutamic acid at position 66 of I-A^b β-chain on the interaction with the B3K506 TCR). These data supported the conclusion that in those cases, the 'parental' amino acid side chain provided an important contact with the TCR that other side chains could not provide.

In other cases, however, substitution of the wild-type amino acid with alanine was tolerated, but substitution with other amino acids was not (**Table 1** and **Fig. 3b–f**). For example, in tests of the binding of YAe62.8 TCR to I-A^b + 3K with various amino acids at position 8, substitution of alanine or glycine for the wild-type lysine had little effect on the binding. However, substitution of glutamine or leucine for the lysine at position 8 of the 3K peptide resulted in substantial reduction in the strength of binding (ΔΔG of 0.9 or 1.1 kcal/mol, respectively). Thus, even though the 'parental' lysine side chain at position 8 of the 3K peptide was irrelevant for binding to the YAe62.8 TCR, some side chains at this position of the peptide substantially reduced the energy of the reaction. This type of 'interface disruption' did not necessarily correlate with exchange of a bulky side chain for a small one or the introduction of a difference in charge (as with the results obtained for lysine at position 8 and YAe62.8, discussed above, and other results in **Table 1** and **Fig. 3b–f**).

Therefore, substitution of the amino acid side chains of the peptide and MHC affects TCR specificity in three ways. Some substitutions

remove important TCR contacts. Less frequently, other substitutions replace a somewhat inhibitory side chain with a side chain that increases the affinity of binding. Finally, some substitutions actively disrupt TCR binding even in cases in which the native side chain was not recognized. We call these 'interface-disrupting' residues.

Interface-disrupting residues contribute specificity

To present the disruptive effect of side-chain substitution as a graph, we calculated the $\Delta\Delta G$ for each MHC-peptide position, comparing the binding energy of the TCR for I-A^b + 3K with the amino acid promoting the most disruption versus the binding energy of the TCR for I-A^b + 3K with alanine at that position. By that calculation, the contribution of recognition of the specific amino acid side chain at that position was obviated and the disruptive effects of the substitution of the new side chain could be measured in isolation. Again, we used a 'color-coded' scheme in which the side chains were assigned colors according to their potential to disrupt TCR binding. At some positions, alanine substitution ablated binding and therefore this calculation could not be made (**Fig. 4f–j**, pink). For each TCR, residues with the greatest potential to cause disruption approximated a 'ring' around the central high-affinity binding core of the I-A^b + 3K complex (**Fig. 4f–j**). In addition, there was a 'hint' that more amino acid residues could be disruptive for interactions with the cross-reactive TCRs than with specific TCRs, although we did not analyze enough TCRs to obtain statistical significance.

Finally, to create a general 'map' for each TCR of how MHC-peptide side chains control specificity, we merged the results of the alanine-scanning experiments and interface-disruption experiments (**Fig. 4k–o**). For sake of clarity, we have emphasized only the MHC-peptide side chains with the biggest effects; that is, those with contributions of 0.8 kcal/mol or more of binding energy or potential disruptive energy. The image that emerged for all the TCRs was of a central region in which the wild-type MHC-peptide amino acid side chains were specifically recognized by the TCRs, with a 'halo' of other amino acids whose side chains contributed much less to the energy of binding. Nevertheless, the 'halo' positions of the MHC-peptide complex that contributed less binding energy could contribute substantial specificity to the recognition of MHC-peptide complexes by the TCR, as substitutions at those positions could disrupt TCR binding.

DISCUSSION

It is well established that thymocytes are selected to mature based on the ability of the TCRs they express to react with moderate affinity with self MHC–self peptide ligands encountered in the thymus[4–7]. The resulting repertoire of mature T cells is very specific to the MHC and peptide, with limited ability of each TCR to recognize variant forms of stimulatory MHC-peptide complexes[35]. It has been suggested that this phenomenon is controlled, unexpectedly, by negative rather than positive selection[19,36].

The idea that negative selection establishes TCR specificity is supported by our data presented here. The two specific TCRs from wild-type C57BL/6 mice were very dependent on high-affinity binding with many side chains of the MHC and peptide. For each, seven or eight of the solvent-accessible side chains of MHC and peptide were 'hot spots' for binding and could not be replaced with alanine or most other amino acids. In contrast, the cross-reactive TCRs from mice in which negative selection was limited were much more 'accepting' of amino acid substitutions in both MHC and peptide, with at most four 'hot spots' for binding.

Some of the changes in free energy of binding caused by substitution with alanine may not accurately reflect the amount of energy directly contributed by the native side chain to the binding reaction. For example, substitution with alanine may allow other amino acids on the surface of I-A^b + 3K to rotate more freely or to adopt a different conformation. A positional change of a neighboring amino acid could change the free energy of binding to the TCR by eliminating a critical bond or requiring the neighboring amino acid to undergo a conformational change in the side-chain rotamer before binding. In addition, it is possible that a reduction in the size of a side chain by substitution with alanine could allow the inclusion of a water molecule at the interface, which, depending on the wild-type amino acid, could affect the energy of binding of the TCR to the MHC-peptide[25,37]. Substituted side chains may also affect the electrostatic 'steering' of the TCR to the MHC-peptide complex. Such an effect would reduce the equilibrium affinity by slowing the 'on rate', regardless of whether the side chain contributed binding energy to the interface. We acknowledge that those are potential caveats to the interpretation of the experiments presented here. However, cross-reactive TCRs use far fewer amino acid side chains to generate their high-affinity binding to I-A^b + 3K than do specific TCRs.

Much of the specificity of a TCR for an MHC-peptide is derived from the recognition of a particular set of side chains at critical TCR contact positions of the peptide[30]. However, TCRs interact to some extent with side chains of the MHC as well, as demonstrated in the solved three-dimensional crystal structures of the binding of TCRs to cognate MHC-peptides[38,39]. The generation of binding energy by TCRs from their interaction with the side chains of the MHC, however, reduces the amount of energy needed from interaction between the TCR and the side chains of the peptide, which suggests that TCRs may not have to be as specific for 'target peptides', as first proposed[40–43]. Theoretically, this is a particular problem for TCRs because their 'target' MHC-peptide complexes are at high concentration on antigen-presenting cells and their affinity for a specific MHC-peptide complex is relatively low[44,45].

Our data presented here have suggested another means whereby specificity may be imparted to the TCR-MHC-peptide reaction. Not only are some MHC-peptide side chains required for the binding reaction, but also others are expressly 'forbidden', even in cases in which the 'parental' side chain does not contribute to the binding affinity. Those residues of the MHC-peptide complex contribute specificity by actively disrupting the ability of the TCR to bind.

Interface-disrupting side chains have a variety of chemical properties, and specificity generated by them is probably not based solely on steric hindrance. Some disruptions are the result of the replacement of small side chains with larger ones; for example, interface disruption occurred with the B3K506 TCR when isoleucine, phenylalanine, aspartic acid or lysine replaced the alanine at position 73 of I-A^b β-chain. However, other side chains are probably disruptive for many biophysical reasons. For example, interface disruption occurred with the B3K506 TCR when leucine or glutamic acid replaced the histidine at position 68 of I-A^b α-chain, but not when phenylalanine or glutamine replaced it, or for the YAe62.8 TCR, when the peptide had substitutions at the position 8. In addition to steric hindrance, therefore, side chains may be disruptive by reducing the ability to exclude water from the interface or by decreasing electrostatic interactions between the TCR and MHC-peptide complex. In addition, some side-chain substitutions may influence the strength of binding through indirect effects. For example, substituted side chains may change the side-chain rotamer of a neighboring amino acid or they may change the position of the backbone of the MHC or peptide.

Because the strength of hydrogen bonds is dependent on the bond angle, relatively small positional changes of the side chain or backbone could have substantial effects on binding affinity.

Interface-disrupting residues can have considerable effects on TCR specificity. The 2W20.4 TCR recognized only two side chains of the peptide with high affinity, whereas the B3K506 and B3K508 TCRs used four side chains of the peptide to contribute high-affinity binding, yet the 2W20.4 T cell was classified as peptide specific. The peptide specificity of the 2W20.4 TCR was in fact generated mainly by interface disruption at position 2 of the peptide. Although a switch to alanine at that position did not change the equilibrium binding affinity, four other substitutions (to leucine, glutamic acid, lysine and tyrosine) led to a ΔΔG of more than 1.7 kcal/mol. In addition, the 'parental' T cell completely fails to be activated by ten different amino acid substitutions at this position[19].

Our finding that certain amino acid side chains can disrupt a TCR-MHC-peptide interface is consistent with other studies[46,47]. For example, for I-E^k plus moth cytochrome *c*, substitution of solvent-exposed side chains of the peptide can eliminate T cell reactivity, whereas replacements at the same position with small amino acids are tolerated[30]. Thus, the contribution of interface-disrupting residues to the overall specificity of the interaction of TCRs with MHC-peptide complexes is probably a general feature of all (or most) TCR-MHC-peptide interactions. Interface-disrupting residues may also explain one of the fundamental features of TCRs: their ability to recognize a subset of amino acids at some positions of the peptide[30,31,34,48].

Each TCR we have studied here has its own unique requirements for amino acid side chains of the peptide and MHC. Of the MHC amino acids, the arginine at position 70 of I-A^b β-chain was the most often recognized, with four or five of the TCRs potentially able to interact with it. In fact, the only side chain that contributed high-affinity binding for all five TCRs was the lysine at position 5 of the peptide. However, even the requirement for lysine at position 5 was not absolute, as B3K506 and YAe62.8 recognized K3 with arginine rather than lysine at position 5 with very little change in affinity. Moreover, a study of T cell reactivity to I-A^b + 3K variants with amino acid substitutions has shown that the 2W1S4 43 T cell is fully activated by essentially every amino acid substitution of the lysine at position 5 of the peptide and the arginine at position 70 of I-A^b β-chain[19]. Thus, contrary to what has been suggested for T cells and has been reported for other receptor-ligand interactions, there do not seem to be any evolutionarily conserved amino acid side chains required for recognition of MHC-peptide complexes by TCRs[49,50].

If there are no conserved side chains required for recognition of MHC proteins by TCRs, how is the TCR repertoire biased toward recognizing MHC? We account for that ability as follows. Before selection, the random rearrangement and pairing of TCRs generates a spectrum of TCRs, many of which can interact with self peptides bound to host MHC through relatively few side chains of the MHC-peptide complex as high-affinity binding sites. Positive selection selects some of those cells. However, because negative selection is critically dependent on the avidity of the TCR-MHC-peptide interaction, and all the MHC proteins encoded by an allele will present similar peptide and MHC main chains and side chains to a TCR, negative selection will 'favor' the elimination of thymocytes that generate too much affinity from those unvarying determinants. Similarly, negative selection will also 'select against' TCRs that generate very large amounts of binding energy from a few side chains of the MHC-peptide in favor of TCRs that generate high-affinity binding energy from many side chains of the interface. Negative selection will allow survival of only those thymocytes bearing TCRs whose binding affinities are derived from interaction with the most variable part of the MHC-peptide complex; that is, the side chains of the peptide.

METHODS

Production of soluble MHC-peptide and TCR proteins and multimers. Soluble I-A^b + 3K and peptide variants and soluble TCRs were produced and purified as described[19]. The sequences of the specific and cross-reactive TCRs are in **Supplementary Table 1**. Multivalent fluorescent TCRs were prepared as described[26]. A complex of biotinylated monoclonal antibody ADO304 to the TCR α-chain constant region and Alexa Fluor 647–streptavidin was formed. The complex was purified by size-exclusion chromatography with a Superdex200 column (GE Healthcare). Fluorescence-labeled ADO304 was mixed with a concentration of soluble TCRs that saturated the antibody-binding sites.

Surface plasmon resonance. The affinity and kinetics of the binding of soluble, monomeric I-A^b + 3K or peptide variants to immobilized TCRs was analyzed by surface plasmon resonance. Approximately 1,000–2,000 resonance units of soluble TCR were captured on the surface of a biosensor flow cell by immobilized monoclonal antibody ADO304. Soluble I-A^b + 3K or peptide variant was injected for 30 s at various concentrations in the range of 0.25–64 μM, depending on the equilibrium affinity of the given TCR–I-A^b + 3K variant interaction. All samples reached equilibrium binding within 10 s. The complex was allowed to dissociate for 1 min between injections. Raw data were corrected for the bulk signal from buffer and I-A^b + 3K by identical injection through a flow cell in which an irrelevant TCR was immobilized. Data were further corrected for the loss of captured TCR during the series of injections based on the observed dissociation rate (k_d) of the TCR from monoclonal antibody ADO304 (about 4.5×10^{-4} per second). Data were analyzed with BIAevaluation software (Biacore). Because the binding kinetics were so rapid, Scatchard analyses of the equilibrium data were used to determine the K_d. The kinetic data were used to determine the dissociation rate of the TCR–I-A^b + 3K variant complexes and the association rate (k_a) was calculated from the K_d and k_d ($k_a = k_d / K_d$). No detectable binding was noted for some TCRs and I-A^b + 3K peptide variants. In those cases, the minimum possible K_d was estimated as 550–650 μM, assuming a maximum possible equilibrium signal of 50 resonance units with the highest concentration of I-A^b + 3K variants tested (32–64 μM) and an R_{max} of 800–1,200 resonance units; that is, the maximum predicted signal at complete binding of I-A^b + 3K to the TCR.

Calculation of Δ*G* and ΔΔ*G* and side-chain inhibition. Free energy of binding (Δ*G*) was calculated with the following equation: $\Delta G = -RT\ln(K_d^{-1})$. For calculation of the amount of binding energy contribution by a 'parental' side chain, the affinity of the 'parental' side chains was compared with that of an alanine substitution at that position. Thus, the 'parental' side chains contribution was determined as follows: ΔΔ*G* = (Δ*G* of alanine substitution) – (Δ*G* of wild-type side chain). For calculation of the amount of potential side-chain inhibition, the following equation was used: ΔΔ*G* of side-chain inhibition = (Δ*G* with the most inhibitory side chain at that position) – (Δ*G* with alanine at that position).

Construction of soluble and membrane-bound TCR and I-A^b + 3K mutant viruses. The soluble TCR and many of the membrane-bound I-A^b + 3K variant baculoviruses have been described[19]. The soluble I-A^b + 3K peptide mutant viruses were made by PCR-based site-directed mutagenesis of the published I-A^b + 3K baculovirus transfer plasmid[26]. New membrane bound I-A^b + 3K mutant viruses were made in a similar way as reported[26].

Staining of Sf9 insect cells expressing I-A^b + 3K variants. Sf9 insect cells were infected for 3 d with baculovirus expressing membrane-bound I-A^b + 3K variants at a multiplicity of infection of 3–10. Virus-infected cells were washed and 1×10^5 cells were stained for 2 h at 25 °C in 20-μl aliquots with 20 μg/ml of TCR-staining reagent and I-A^b (monoclonal antibody 17/227; provided by J. Freed, National Jewish Medical and Research Center, Denver, Colorado). Cells were then washed and were immediately analyzed by flow cytometry. Each I-A^b + 3K variant was stained three independent times. To maximize reproducibility of TCR multimer staining fluorescence, we repeated

the experiments on subsequent days with the same preparation of TCR multimer–staining reagent and the same flow cytometer and settings.

Generation and analysis of fluorescent TCR multimer versus affinity standard curve. We used fluorescence-labeled soluble TCR multimers to stain cells expressing I-A^b + 3K or its variants at potential TCR contacts of the peptide or MHC. We began this analysis with the YAe62.8 and B3K506 TCRs. Insect cells expressing I-A^b + 3K but not I-A^b plus an irrelevant peptide (Eα) bound the multimeric YAe62.8 and B3K506 TCRs. Furthermore, there was a direct relationship between the expression of I-A^b on the cells and the extent of TCR binding. In comparisons of cells with the same high expression of I-A^b, the extent of TCR binding measured by MFI was similar for those two TCRs, correlating with their similar affinities for I-A^b + 3K measured by surface plasmon resonance. We then extended that analysis to the variants of 3K peptide for which we had made surface plasmon resonance affinity measurements for those two TCRs. There was a direct relationship between the measured affinity (K_a) and TCR multimer staining (MFI). Finally, we included in the analysis the three other TCRs, combining all of the data to create a 'standard curve' relating affinity to multimer staining.

The standard curve was generated by the least-squares method of curve fitting with a third-order polynomial to generate an average 'titration' curve from all 'titration' data. We analyzed the data, assuming that the curves for each TCR had the same shape but could have different intercepts, using the program 'MKassay' (available on request). The algorithm generates parallel curves for each TCR analyzed with the data from all five TCR analyzed.

Note: Supplementary information is available on the Nature Immunology website.

ACKNOWLEDGMENTS

We thank G. Murphy and S. Dai for discussions. Supported the US Public Health Service (AI-17134, AI-18785, AI-52225, AI-22295 and P30CA046934).

AUTHOR CONTRIBUTIONS

E.S.H., F.C., J.W. and J.W.K. did the experiments; P.M. made the cells from which the TCRs were derived; and E.S.H., P.M. and J.W.K. wrote the paper with the help of J.W. and F.C.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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