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How the T Cell Repertoire Becomes Peptide and MHC Specific

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Summary

T cells bearing αβ T cell receptors (TCRs) recognize antigens in the form of peptides bound to class I or class II major histocompatibility proteins (MHC). TCRs on mature T cells are usually very specific for both peptide and MHC class and allele. They are picked out from a precursor population in the thymus by MHC-driven positive and negative selection. Here we show that the pool of T cells initially positively selected in the thymus contains many T cells that are very crossreactive for peptide and MHC and that subsequent negative selection establishes the MHC-restriction and peptide specificity of peripheral T cells. Our results also suggest that germline-encoded TCR variable elements have an inherent predisposition to react with features shared by all MHC proteins.

Introduction

T cells bearing αβ T cell receptors (TCRs) recognize invading organisms in the form of peptides derived from the invader bound to major histocompatibility proteins (MHC) of their host (Townsend and Bodmer, 1989; Unanue, 1984). The genes coding for TCRs are produced by gene rearrangement during T cell development in the thymus. This process creates receptors that are very different in sequence from one T cell to another. However, all TCRs include a Vα, Jα, Vβ, Dβ, and Jβ segment, each selected from a set of germline genes. Only the junction regions between each of these segments are not germline encoded (Davis and Bjorkman, 1988). On the whole, these genes do not vary dramatically between different members of a species. Thus, each individual has the ability to produce T cells bearing approximately the same (albeit very large) collection of TCRs.

In contrast, the MHC proteins to which these TCRs bind vary dramatically in sequence from one individual of a species to another. For example, in man, more than 800 alleles of the MHC class I and 600 alleles of MHC class II proteins are known (Robinson et al., 2003). The matter is also complicated by the fact that, within each individual, some T cells react with foreign peptides bound to MHC class I proteins, whereas other T cells react with peptides bound to MHC class II proteins (Katz et al., 1975; Zinkernagel and Doherty, 1974). These two sets of T cells are also usually distinguishable by the presence on their surfaces of CD8 or CD4, which bind to MHC class I or class II, respectively. They also differ in their functions. $CD8^+$, class I-restricted T cells are usually cytotoxic, whereas $CD4^+$, class II-restricted T cells often act to help the responses of other leukocytes (Goldrath and Bevan, 1999; Starr et al., 2002; von Boehmer et al., 2003).

These observations have led to two longstanding problems in immunology. First, how can a relatively limited collection of TCR Vα, Jα, Vβ, Dβ, and Jβ segments produce a set of TCRs that, in any individual, is quite specific, both for peptide and the alleles of MHC expressed in that individual? This property is usually thought to be the result of MHC-driven positive selection, a phenomenon that occurs during thymocyte development. Positive selection allows conversion to mature T cells of only those thymocytes that bear TCRs with low but appreciable affinity/avidity for MHC + peptide combinations present in the thymus (Goldrath and Bevan, 1999; Starr et al., 2002; von Boehmer et al., 2003). However, two other events that occur in the thymus might bear on the specificity of mature T cells: (1) death by neglect, an event whereby thymocytes with no appreciable affinity/avidity for MHC + peptide combinations in the thymus die (Berg et al., 1989; Scott et al., 1989), and (2) negative selection, an event whereby thymocytes bearing TCRs with high affinity/avidity for ligands in the thymus also die (Kappler et al., 1987; Kisielow et al., 1988). Negative selection prevents the peripheralization of most autoreactive cells (Mathis and Benoist, 2004). Because of the demands of positive and negative selection, only a few of all possible TCRs are expressed on mature T cells in any given animal.

The second question deals with the problem of how the relatively conserved set of TCR polypeptides can create TCRs that can react with all the different alleles of MHC that might be present in different individuals of the species and, most dramatically, with MHC proteins of both types, class I and class II. The explanation for the class I versus class II problem might lie partly in the coexpression of CD8 versus CD4 by T cells since the binding of CD8 to class I and CD4 to class II certainly increases the avidity with which an individual T cell reacts with target cells bearing class I or class II. However, this cannot be the only solution to the problem since T cells are known which can react with their MHC + peptide target independently of their coexpression of CD8 or CD4 (Goldrath and Bevan, 1999; Starr et al., 2002; von Boehmer et al., 2003). Likewise, for some rare T cells, expression of CD4 or CD8 is not concordant with the class of MHC with which they react (Kirberg et al., 1994). In extreme examples of this unpre-

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dictability, T cells have been described that react with both MHC class I and class II (Schilham et al., 1986).

Another factor might contribute to these phenomena. Many years ago, Nils Jerne suggested that lymphocyte receptors might be intrinsically biased toward recognition of MHC (Jerne, 1971); that is, TCR germline-encoded elements might have some evolutionarily selected means of reacting with MHC proteins regardless of MHC allele or class. However, the mechanism whereby such global recognition might occur is difficult to imagine, given the variability of MHC proteins and the fact that it has not been manifest in the solved structures of TCRs bound to their MHC + peptide targets (Housset and Malissen, 2003).

In this paper we studied TCRs from T cells that have been less stringently negatively selected than those from normal animals. Among this collection, we found individual TCRs that were often very crossreactive between different alleles of MHC and could react with both class I and class II MHC proteins bound to peptides. Many of these TCRs reacted with MHC and peptide in a fashion that was relatively independent of the surface-exposed amino acid side chains of the MHC and peptide. These findings led us to two conclusions. First, they indicate that negative selection is involved in focusing T cell responses on foreign peptides bound to self rather than foreign MHC alleles. Second, they suggest that germline-encoded TCR segments are predisposed to react with a feature(s) shared among all MHC proteins, e.g., the helix backbones, regardless of their amino acid side chains.

Results

Negative Selection Controls the Specificity of T Cells for Foreign Peptides

We began with a study of how the peptide specificity of mature T cells reactive to a foreign peptide is altered by limiting negative selection. We compared three types of mice. First, we used wild-type C57BL/6 mice in which the combination of IA^b and numerous mouse self-peptides leads to the normal negative selection of many thymocytes. Second, we used mice genetically manipulated to express IA^b bearing just a single peptide (IA^b-SP mice) (Ignatowicz et al., 1996). In this case, although many T cells are positively selected in the thymus, negative selection is severely limited because there is only one IA^b + peptide combination present. Finally, we used mice that were devoid of MHC class II ($CL2^{-/-}$ mice).

We immunized these mice with dendritic cells presenting IA^b covalently bound to a foreign peptide, 3K. We then enriched the responding IA^b + 3K-reactive T cells and, for ease of analysis, converted them into T cell hybridomas. These cells were then assayed for their ability to respond to a set of fibroblasts, each expressing IA^b covalently bound to the 3K peptide or to peptides with amino acid substitutions at each of the 5 3K amino acid residues most accessible to TCRs (Figure 1A and Figure S1 in the Supplemental Data available with this article online) (Huseby et al., 2003; Liu et al., 2002). By this means, we were able to identify the amino acid side-chain requirements for T cell activation at each position of 3K.

An example of the pattern of response of a T cell hybridoma from each type of immunized mouse is shown in Figures 1B–1D. All three hybridomas responded to the 3K peptide (red bars), and none responded in the absence of antigen-presenting cells. The response of the T cell hybridoma isolated from a C57BL/6 mouse was unaffected by changes in the glutamic acid at position P–1 of the peptide (Figure 1B). Its response was affected by some substitutions of the lysine at P8 and more dramatically by substitutions of the glutamine at P2. Most substitutions at P3 and P5 reduced or eliminated the response of the hybridoma. Thus, the TCR on this hybridoma was focused primarily on peptide residues P2, P3, and P5. Similar analyses of the responses of the T cell hybridoma isolated from an IA^b-SP mouse revealed that the TCR on this cell was focused almost exclusively on the peptide amino acid at P5 (Figure 1C). The TCR on the single hybridoma from a $CL2^{-/-}$ mouse had a specificity for peptide similar to that of the C57BL/6 T cell hybridoma (Figure 1D). However, the overall reactivity of this last TCR was probably underestimated with respect to the reactivities of the other two TCRs illustrated here because the T cell hybridoma from the $CL2^{-/-}$ mouse, unlike all other hybridomas examined, bore low levels of the costimulatory protein, CD4.

We cannot of course tell from these three TCR analyses whether the effects of changes in the peptide reflect direct interactions between the peptide amino acid itself and the TCR in question or reflect indirect interactions in which the changed peptide amino acid masks or changes the configuration of some other IA^b + peptide residue that is itself contacting the TCR. Nevertheless, these changes do reflect the relative ability of each TCR to interact with IA^b bound to diverse peptide ligands.

A summary of the results for all the T cells and peptides tested is shown in Figure 2. To simplify presentation of these analyses, we used a color-o-metric scheme to indicate those substituted peptides that activated the T cells as efficiently as the wild-type 3K peptide did versus those that gave partial or no activation. Control experiments indicated that all of the T cell hybridomas responded well to the immunizing 3K antigen. In addition, many T cells from IA^b-SP mice reacted with IA^b + self-peptides, while T cells from C57BL/6 T cells were, of course, tolerant of these combinations (data not shown).

The TCRs on 3K-specific T cells isolated from C57BL/6 mice were very sensitive to changes in at least three positions of the 3K peptide (Figures 1B and 2A). Conversely, the TCRs on T cells derived from IA^b-SP or $CL2^{-/-}$ mice were much more variable in their requirements (Figures 1C, 1D, 2C, and 2D). Some had specificities like those of TCRs from C57BL/6 and were affected by changes in the peptide sequence at three or more of the peptide positions tested. Others were much more degenerate in their specificity for peptide and were affected by changes in only one of the peptide residues, although the peptide residue involved could be at one of several positions, P3, P5, or P8.

To determine whether the high degree of peptide degeneracy seen in some T cells isolated from IA^b-SP mice was the result of positive selection on a single peptide or was due to a lack of negative selection on

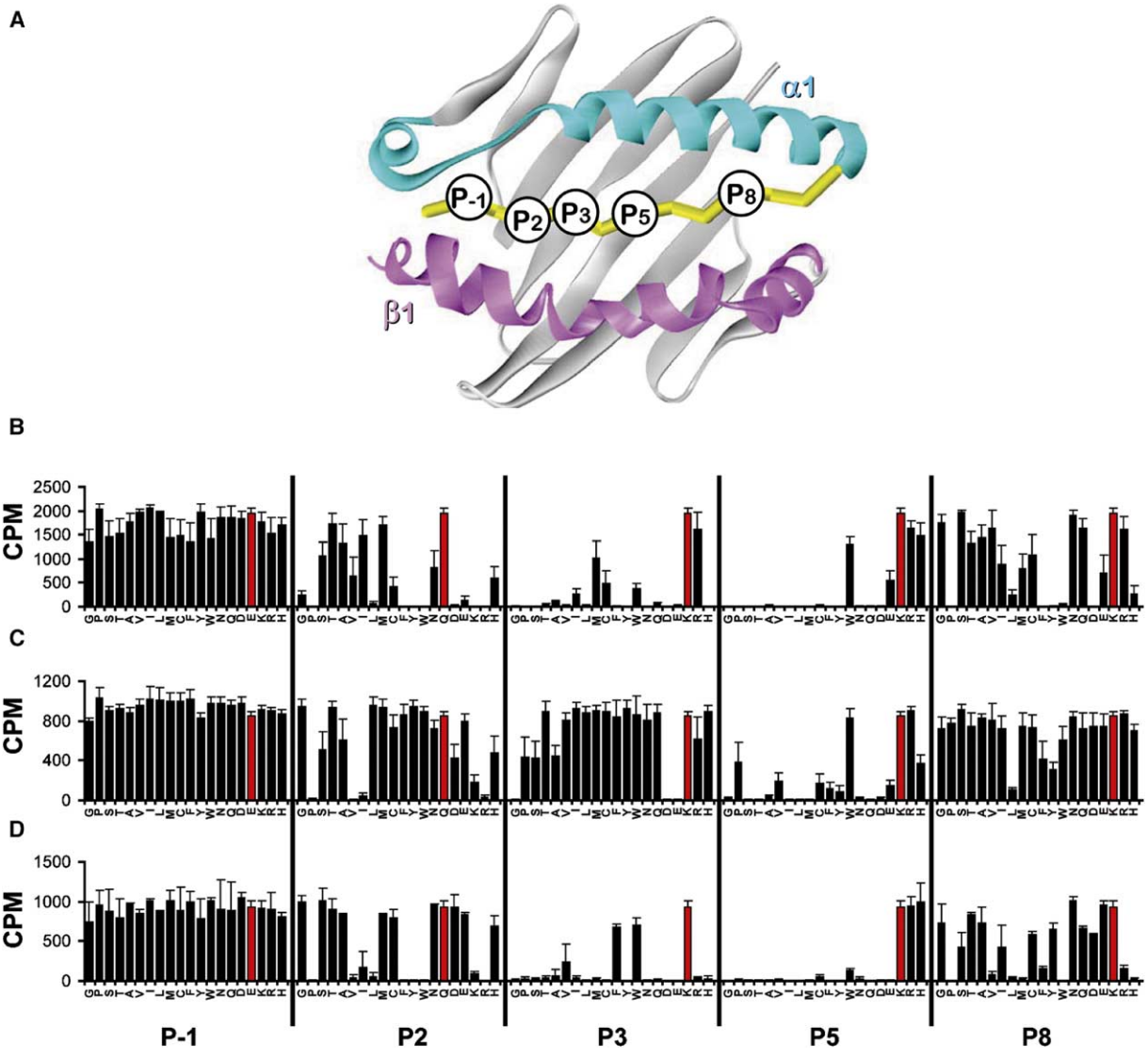


Figure 1. Identification of Single-Amino Acid-Variant Activating Ligands for the IA^b + 3K-Specific T Cells

(A) The structure of the face of IA^b + 3K that is exposed to TCRs, showing the peptide amino acids that were mutated for these studies (Liu et al., 2002).

(B–D) The (B) B3K 506 (isolated from a C57BL/6 mouse), (C) YAe5 62.8 (isolated from an IA^b-SP mouse), and (D) 3K-36 (isolated from a $CL2^{-/-}$ mouse) T hybridoma cells were stimulated with APC expressing IA^b + 3K or all 19 single-amino acid substitutions at each of the five potential TCR contact positions. Error bars show the standard deviations of at least three independent assays.

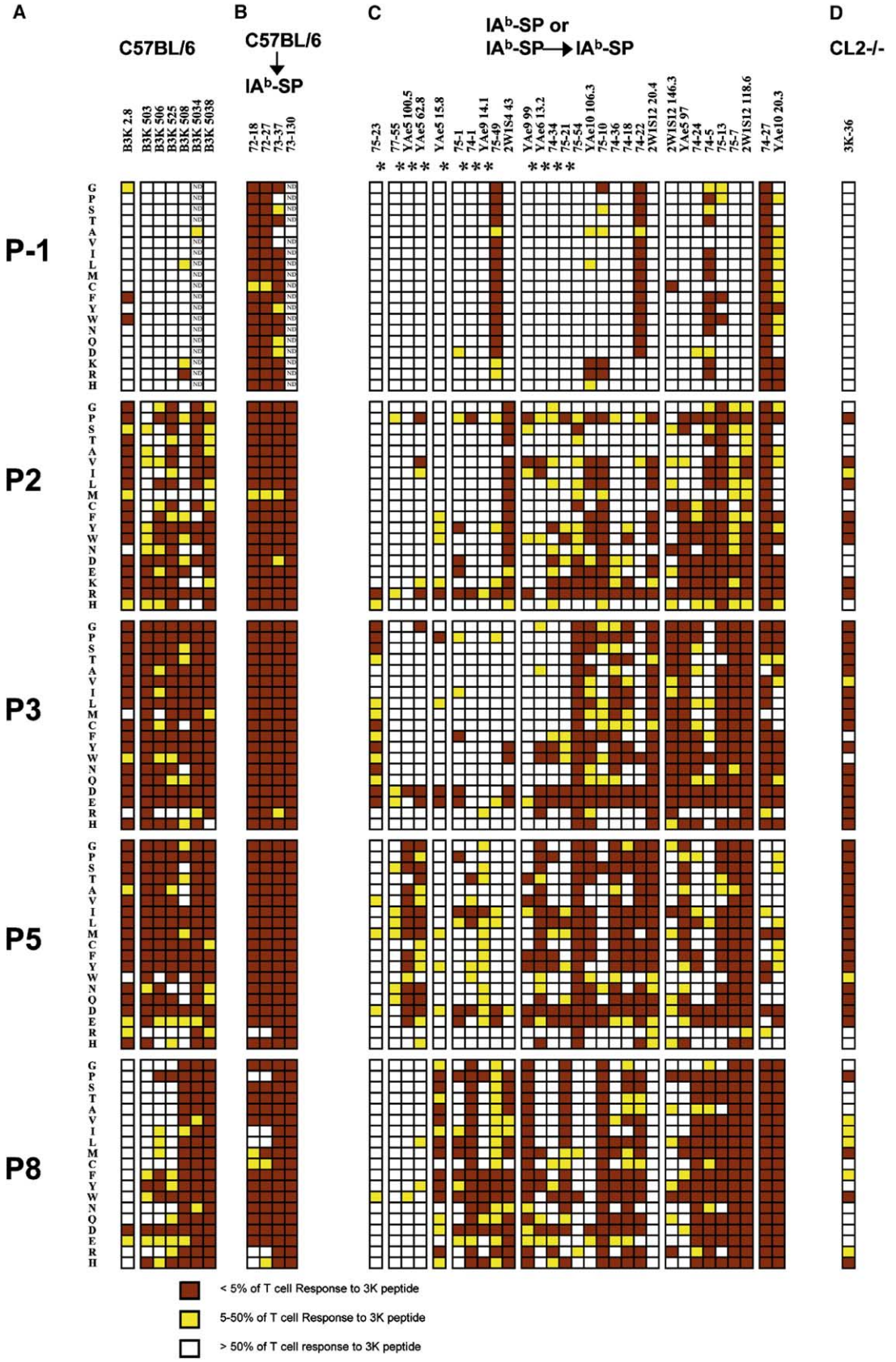
the diverse set of self-peptides presented by normal self-MHC molecules, we constructed bone-marrow (BM) chimeras in which either C57BL/6 or IA^b-SP BM cells were used to reconstitute lethally irradiated IA^b-SP mice. When IA^b-SP mice were reconstituted with IA^b-SP BM, again, many of the T cells derived from these mice were critically dependent upon only a single amino acid position for complete T cell activation. However, when constricted by normal negative selection by C57BL/6 BM cells, the ensuing T cell repertoire positively selected on IA^b-SP was highly peptide specific (Figures 2B and 2C).

The overall peptide crossreactivity of the T cells negatively selected on IA^b-SP was highly significantly different ($p < 0.005$) from that of T cells that had been negatively selected on C57BL/6 by a Mann-Whitney test. A Student's t test showed that 12 of the IA^b-SP T cells had crossreactivities that were significantly different from that of the population of T cells negatively selected on C57BL/6 ($p < 0.05$). These T cells are indicated by asterisks in Figure 2.

Thus, overall, the exquisite specificity for peptides of T cells in normal mice is controlled by negative selection because negative selection deletes T cells with degenerate reaction with peptide.

TCRs that Have Undergone Limited versus Extensive Negative Selection Have Overlapping Avidities and Relative Affinities for IA^b + 3K

We were concerned that the TCRs from IA^b-SP mice might have higher affinity for IA^b + 3K than the TCRs that had undergone normal negative selection on


A
B
C
D
C57BL/6
C57BL/6
IA^b-SP
IA^b-SP or
IA^b-SP → IA^b-SP
CL2-/-
B3K 2.8
B3K 503
B3K 506
B3K 525
B3K 508
B3K 5034
B3K 5038
72-18
72-27
73-37
73-130
75-23
77-55
YAe5 100.5
YAe5 62.8
YAe5 15.8
75-1
74-1
YAe9 14.1
75-49
2W1S4 43
YAe9 99
YAe6 13.2
74-34
75-21
75-54
YAe10 106.3
75-10
74-36
74-18
74-22
2W1S12 20.4
2W1S12 146.3
YAe5 97
74-24
74-5
75-13
75-7
2W1S12 118.6
74-27
YAe10 20.3
3K-36
P-1
P2
P3
P5
P8
G P S T A V I L M C F Y W N Q D K R H
G P S T A V I L M C F Y W N D E K R H
G P S T A V I L M C F Y W N Q D E R H
G P S T A V I L M C F Y W N Q D E R H
G P S T A V I L M C F Y W N Q D E R H
< 5% of T cell Response to 3K peptide
5-50% of T cell Response to 3K peptide
> 50% of T cell response to 3K peptide

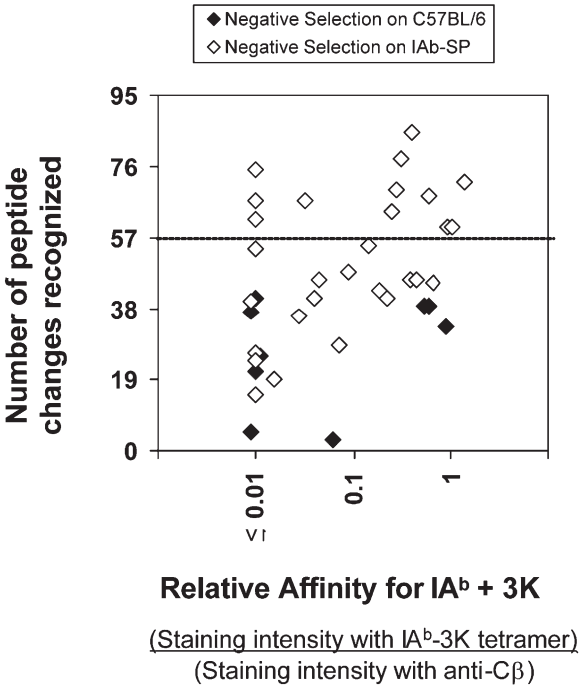


Figure 3. The T Cell Repertoire in Negative-Selection-Limited Mice Contains High- and Low-Affinity, Peptide-Specific, and Peptide-Degenerate TCRs

The number of single-amino acid changes giving complete T cell activation from Figure 2 were counted and plotted against relative TCR affinity for the IA^b + 3K immunizing antigen determined by IA^b + 3K tetramer staining. T cells negatively selected on IA^b-SP (◇) have a range of affinity and peptide degeneracy that overlaps that of T cells from mice negatively selected on wt IA^b (◆). The dashed line indicates TCRs with degeneracy significantly ($p < 0.05$) different from that of TCRs isolated from C57BL/6 mice.

C57BL/6 cells and that this higher affinity might account for the extensive peptide crossreactivity of some of the T cells from the IA^b-SP mice. To check this, we compared the affinities of the two populations of T cells for IA^b + 3K by two methods: comparison of their abilities to bind tetramers composed of the IA^b + 3K ligand (Figure 3) (Crawford et al., 1998) and ease of inhibition of their response to IA^b + 3K by anti-IA^b antibodies (Figure S2). The results of the two methods agreed well. There were no significant differences in the affinities of TCR isolated from C57BL/6 versus IA^b-SP mice. Moreover, among the T cells from IA^b-SP mice, the T cells that had the most degenerate recognition of the 3K peptide (above the dotted line) did not have a range of affinities for IA^b + 3K different from that of the less degenerate T cells (Figure 3).

Negative Selection Affects the Specificity of T Cells for Self-MHC

Since many of the T cells that developed in IA^b-SP mice were very degenerate in peptide specificity, we reasoned that much of the binding energy needed for their activation came from interaction of the TCRs with the MHC rather than the peptide portion of their ligand. For example, perhaps these T cells were focused on the side chains of the most exposed amino acids on the tops of the α helices of the α and β chains of IA^b. Therefore, we expressed IA^b + 3K proteins in which each of the five most exposed potential TCR contacts on the IA^b α and β chain α helices had been randomly mutated to a number of different amino acids (Figures 4 and 5A). Each mutant was tested for its ability to present the 3K peptide to the bank of T cell hybridomas. A few mutations (most often proline or glycine) led to poor IA^b expression. These were eliminated from further analysis. A summary of the results is shown in Figure 4, again with a color-o-metric scale distinguishing those IA^b mutations that had no effect on activation of the T cell hybridoma in question from those that partially or completely eliminated T cell activation.

In some cases, T cell activation absolutely required a particular side chain, and even a highly conserved amino acid substitution was not tolerated. For example, the responses of almost all the T cell hybridomas were severely compromised by any changes of IA^bβ 70R, except to an L (Figures 4E–4H). In other cases, some substitutions were allowed while others were not, and these effects depended on the T cell hybridoma tested. Additionally, some side chains appeared to be completely irrelevant for activation of the T cell being studied. For example, the responses of some T cell hybridomas were unaffected by all changes in IA^bβ 66E (Figures 4F and 4G).

All of the T cells from C57BL/6 mice, as well as from chimeric mice in which T cells which had been positively selected on IA^b-SP and negatively selected on C57BL/6 BM, were affected by at least 2 IA^bα changes, often IA^bα 55D and 61Q, with spotty effects of changes in other IA^bα residues. A range of effects was seen for T cells from IA^b-SP mice. Some T cells were sensitive to changes in all amino acids tested, while others were sensitive to very few alterations in IA^bα.

As far as changes in IA^bβ were concerned, as noted above, all but one T cell were affected by changes in IA^bβ 70R. All T cells from C57BL/6 and chimeric mice undergoing negative selection on C57BL/6 BM were af-

Figure 2. The Relative Level of Peptide Specificity Is Determined by the Extent of Negative Selection

Individual IA^b + 3K-specific T cell hybridomas isolated from (A) C57BL/6 or (B) chimeric mice in which IA^b-SP mice were reconstituted with C57BL/6 BM, (C) IA^b-SP mice or IA^b-SP mice reconstituted with IA^b-SP BM, or (D) $CL2^{-/-}$ mice were activated with APC expressing single-amino acid variants of IA^b + 3K. A color-o-metric scale is used to distinguish fully activating ligands from substitutions that reduced or eliminated T cell responses. Because proliferation is a logarithmic expansion, a 50% rate of proliferation was used to distinguish fully activating ligands from partial or null ligands. Data were generated by averaging results from three independent experiments that had been internally normalized to the response to IA^b + 3K. A * indicates that the peptide contacts for that TCR are significantly more degenerate than those of the population of TCRs negatively selected on C57BL/6 TCRs using a one-tailed Student's t test ($p < 0.05$).

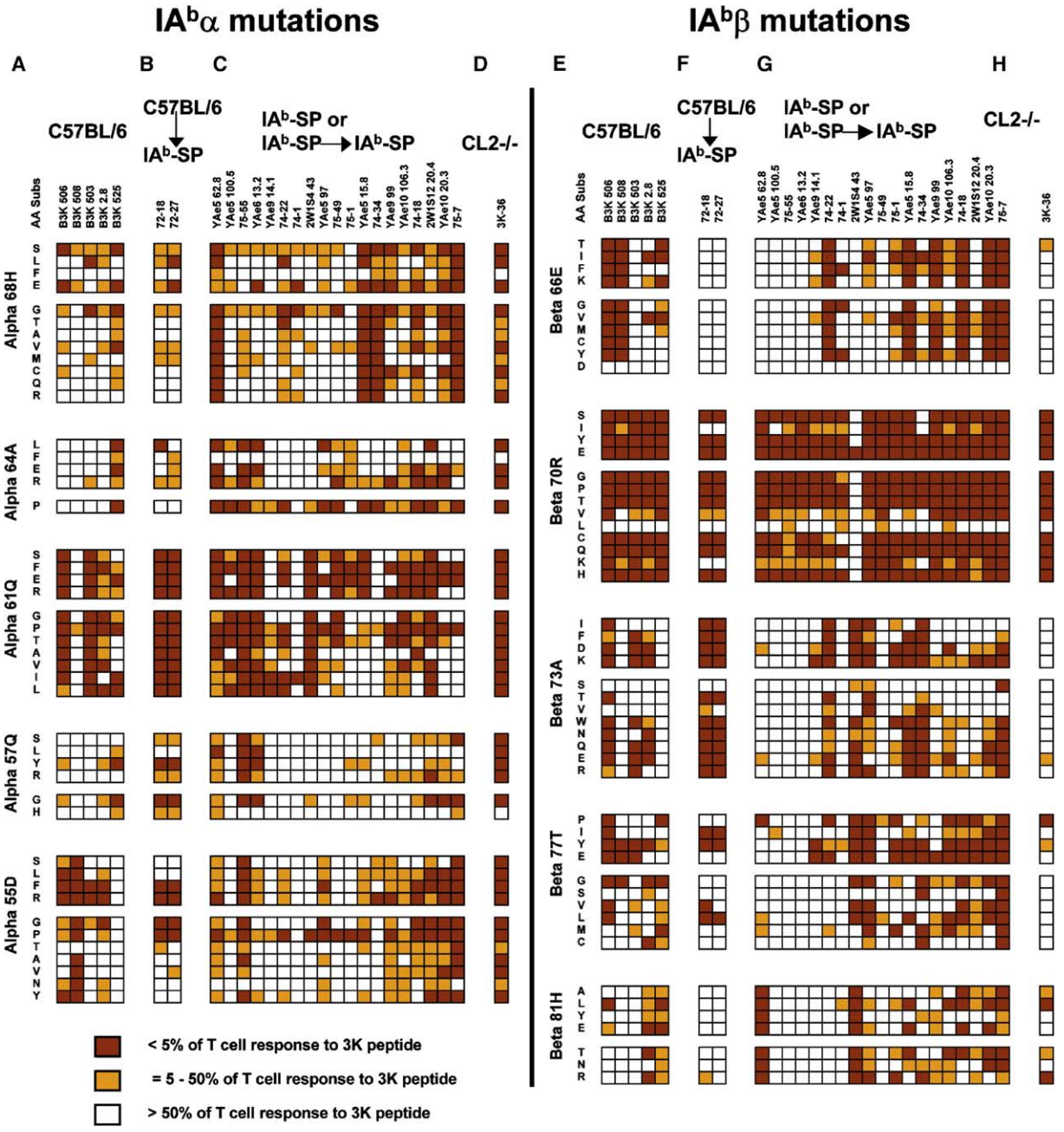


Figure 4. Identification of MHC Contacts for 3K-Specific T Cells

T cells from (A and E) C57BL/6 mice, (B and F) C57BL/6 BM into IA^b-SP chimeric mice, (C and G) IA^b-SP or IA^b-SP BM into IA^b-SP chimeric mice, and (D and H) CL2$^{-/-}$ mice were challenged with IA^b + 3K on which five potential TCR contacts on the IA^b α (A–D) and IA^b β (E–H) chains had been mutated. The four amino acid substitutions above the break in each section indicate the four most chemically distinct amino acids for each position tested. Data were generated by averaging three independent experiments, each of which had been internally normalized to the response to IA^b + 3K.

fected by multiple changes elsewhere in IA$^b\beta$. Interestingly, a subset of T cells from the IA^b-SP animals were resistant to changes in IA$^b\beta$ at any of the other positions tested.

Footprints for Binding of the TCRs to IA^b + 3K

To summarize the results of our experiments thus far, we generated color-coded "footprints" of the T cell responses on a representation of IA^b + 3K (Figure 5). In this figure, for each T cell, 15 rectangles represent the 10 amino acids of the MHC and the 5 amino acids of the peptide and are arranged in a 3 × 5 grid that roughly represents their orientations on the surface of the MHC-peptide complex. The rectangles are colored according to the sensitivity of the T cell to mutations at this position: dark blue, very sensitive; light blue, some-

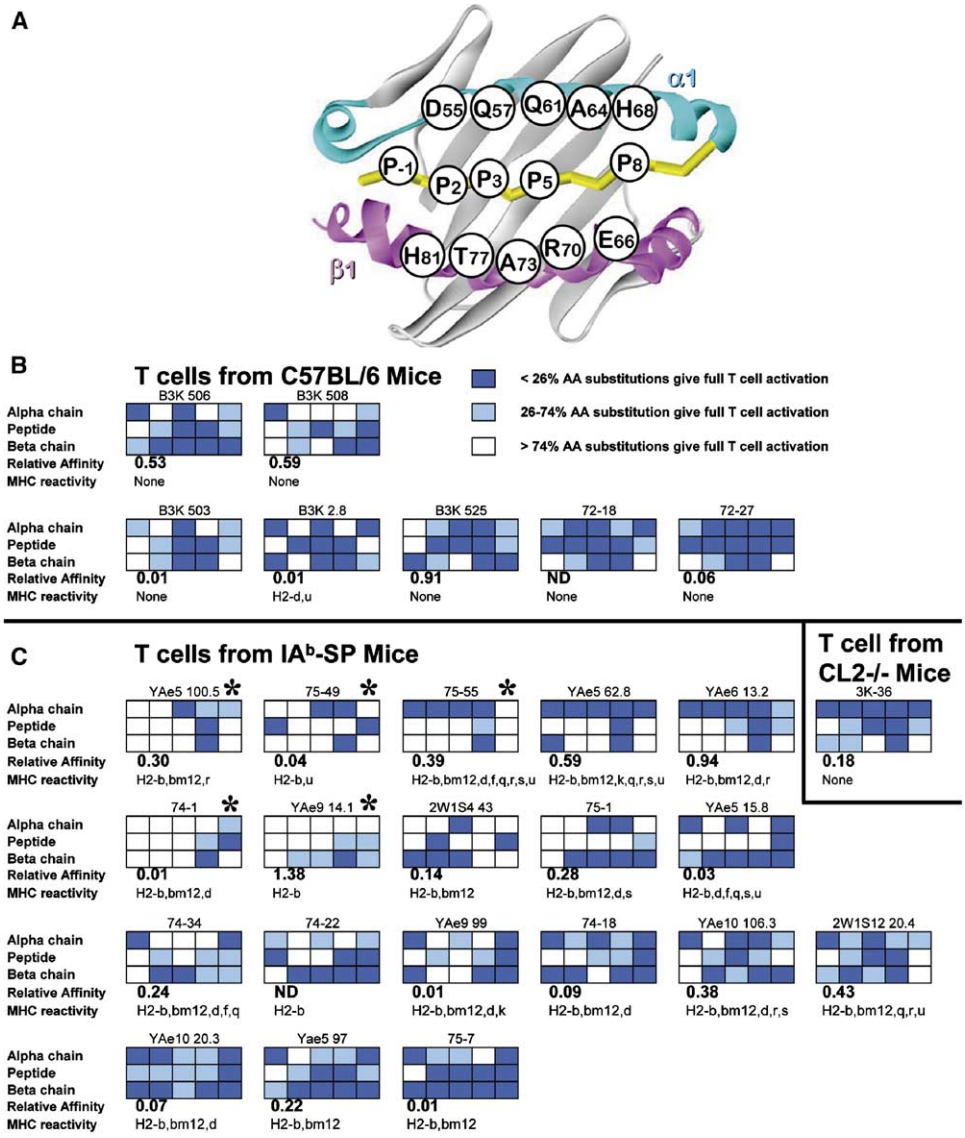


Figure 5. The TCR Repertoire in Mice in which Negative Selection Is Limited Contains TCRs that Interact in Unconventional Ways with MHC Proteins

(A) The structure of the face of IA^b + 3K that is exposed to TCRs showing the IA^b and peptide amino acids that were mutated for these studies (Liu et al., 2002).

(B and C) A footprint of TCR-MHC-peptide interaction for each TCR was constructed in which each MHC or peptide position mutated in Figures 2 and 4 was given a color depending upon the number of amino acid substitutions that retained the ability to completely activate the IA^b + 3K-specific T cell under examination. A * indicates that the MHC + peptide contacts for that TCR are significantly less than those of the population of TCRs negatively selected on C57BL/6 BM using a one-tailed Student's t test ($p < 0.05$).

what sensitive; and white, insensitive. See the figure legend for more details.

T cells from C57BL/6 mice were quite specific for multiple amino acid side chains of the IA^b α and β chains as well as of the activating peptide (Figure 5B). This result is in agreement with published mutagenesis experiments and crystal structures of TCR-MHC-peptide complexes (Housset and Malissen, 2003). In contrast, TCRs from IA^b-SP and $CL2^{-/-}$ mice displayed a range of requirements for interactions with both MHC and peptide side chains (Figure 5C). Some of these TCRs were very specific for amino acid residues of both helices of IA^b and the peptide, much like the TCRs from C57BL/6 mice. However, there were other patterns of MHC interaction, especially among the peptide-promiscuous T cells. Some (YAe5 100.5, 75-49, 75-55, YAe13.2, 74-1) interacted with less of the IA^b β chain helix, while others (74-1, YAe9 14.1, 2W1S4 43) interacted with less of the α chain helix than TCRs from C57BL/6 mice ($p < 0.05$). In addition, five of the TCRs from IA^b-SP animals (YAe5 100.5, 75-49, 75-55, 74-1, and YAe9 14.1) made significantly less contact with the side chains of the amino acids of both IA^b and the 3K peptide than did T cells from normal mice ($p < 0.05$). A

Table 1. Affinities and Binding Kinetics of Specific and Degenerate TCRs to IA^b + 3K

TCR	Origin	MHC + Peptide Crossreactivity	K_D (μM)	k_d (1/s)	$t_{½}$ (s)	k_a (1/s × M)
B3K 506	C57BL/6	none	10.7	1.3	0.5	122,000
B3K 508	C57BL/6	none	41.3	0.43	1.6	10,400
75-55	IA^b-SP	MHC + peptide	17.2	0.64	1.1	36,900
74-1	IA^b-SP	MHC + peptide	>400	ND	ND	ND
YAe5 62.8	IA^b-SP	peptide	12.4	1.5	0.5	121,000
75-1	IA^b-SP	peptide	14.1	1.8	0.4	128,573
2W1S12 20.4	IA^b-SP	none	16.9	0.65	1.1	38,700
3K-36	$CL2^{-/-}$	none	20.7	0.34	2.0	16,500

The categorization of MHC + peptide crossreactivity was established in Figures 2 and 5. The K_Ds were calculated from Scatchard analysis.

Mann-Whitney test showed that, overall, the crossreactivity for MHC + peptide of the T cells from IA^b-SP mice was significantly higher than that of the T cells that had been negatively selected on C57BL/6 ($p < 0.05$).

These results indicate that the peptide promiscuous T cells that develop in IA^b-SP mice do not compensate for their reduced interaction with the peptide amino acid side chains by increasing their interaction with the side chains of amino acids on the tops of the MHC α helices.

TCRs with Degenerate Reactivity for MHC + Peptide and TCRs that Are Very Specific for MHC + Peptide Have the Same Range of Affinities and Binding Kinetics for IA^b + 3K

As in our studies on degeneracy of peptide recognition, above, we were concerned that the TCRs that have very degenerate reaction with MHC + peptide might have different affinities or kinetics of binding than the TCRs that are very specific for MHC + peptide do. The relative affinities of the TCRs, derived from the IA^b + 3K tetramer binding data (Figure 3), are therefore indicated in Figure 5. Clearly there is no relationship between the relative affinity for IA^b + 3K and the crossreactivity of the TCR.

To check whether the distinguishing feature might lie in the kinetics with which the TCRs react with IA^b + 3K, we made soluble versions of some of the TCRs and measured their affinities and kinetics of binding to IA^b + 3K by surface plasmon resonance. For these studies, we chose two TCRs from C57BL/6 mice and two TCRs from IA^b-SP or $CL2^{-/-}$ mice that were quite specific for both MHC and peptide, two TCRs from IA^b-SP mice that were significantly crossreactive for peptide (Figure 2), and two TCRs from IA^b-SP mice that were significantly crossreactive for both MHC and peptide (Figure 5). Seven of these TCRs had very similar relative affinities for IA^b + 3K as measured by tetramer binding or antibody inhibition (Figures 3 and 5 and Figure S2). Therefore, to increase the range of affinities tested, one of the MHC + peptide-crossreactive TCRs was selected because, in the previous tests, it had very low relative affinity for IA^b + 3K.

As expected, the K_Ds of the seven TCRs that had similar relative affinities in our previous tests were again similar when measured by surface plasmon resonance (Table 1) and were consistent with previous biophysical studies of TCRs binding to agonist ligands (Krogsgaard and Davis, 2005) The TCR of 74-1, which had low relative affinity in previous tests, proved also to have a poor affinity as measured by surface plasmon resonance. For the seven TCRs for which such measurements could be taken, the k_ds, k_as, and $t_{½}$s of their interaction with IA^b + 3K were within a similar range whether the TCRs were quite specific or very crossreactive. For example, the very crossreactive TCR 75-55 had a k_d of 0.64/s, a k_a of 36,900/s × M, and a $t_{½}$ of 1.1. These measurements were almost identical to those of the very specific TCR, 2W1S12 20.4.

Thus, the difference between the very degenerate and very specific TCRs does not lie in their affinities or kinetics of binding to their MHC + peptide ligand.

Negative Selection Affects Reactivity with Allogeneic MHC

To characterize the MHC reactivity of T cells isolated from C57BL/6 and IA^b-SP mice more fully, we screened the IA^b + 3K-specific T cells for their ability to react with self-peptides presented by nine different MHC alleles: H-2b, bm12, d, f, k, q, r, s, and u (data not shown). Those combinations that resulted in stimulation are listed for each hybridoma in Figure 5. While only 1 of 7 T cells from C57BL/6 or chimeric IA^b-SP mice undergoing negative selection on C57BL/6 BM responded to any MHC proteins of these alleles (2 total responses out of 63 possibilities), all 19 T cells isolated from IA^b-SP mice showed response to MHC proteins of some allele. Additionally, some T cells from IA^b-SP mice responded to MHC proteins of most of the alleles tested, 8 of 9 alleles for the 75-55 T cell and 7 of 9 MHC alleles for the YAe5 62.8 T cell. In total, the 19 T cells tested from IA^b-SP mice responded to MHC proteins in 70 of a possible 171 combinations tested. The 3K-36 T cell isolated from a $CL2^{-/-}$ mouse did not show any MHC reactivity. However, as mentioned above, this T cell hybridoma expresses low levels of CD4 and no CD8, and this lack of coreceptor signal may have prevented some responses. Hence, foreign-antigen-specific T cells have a florid propensity for allo- and self-MHC reactivity when not constricted by proper negative selection.

Properties of T Cells with Both MHC Class I and MHC Class II Specificity

In the course of examining TCR-transgenic (TCR-Tg) mice constructed from some of the IA^b + 3K-reactive T cells in this study, we found evidence, surprisingly, that

not only do T cells developing under limiting negative selection have a propensity to be peptide and MHC-allele degenerate but also that some recognize both MHC class I and class II. We constructed five TCR-transgenic mice and bred them onto the Rag1$^{-/-}$ background. Two of the TCRs were from C57BL/6 mice, two were from IA^b-SP mice, and one was from CL2$^{-/-}$ mice (Figure 6).

As expected, T cells bearing the IA^b + 3K-reactive TCRs from C57BL/6 mice (506 and 508 TCR-Tg derived from the B3K 506 and B3K 508 T cell hybridomas) developed preferentially into mature CD4$^+$ T cells in mice bearing wild-type IA^b but failed to develop past the immature double-positive stage in CL2$^{-/-}$ mice (Figures 6A and 6B). In contrast, thymocytes bearing TCRs from one of the T cells from IA^b-SP mice (20.4 TCR-Tg derived from the 2W1S12 20.4 T cell hybridoma) failed to mature to the single-positive stage in wild-type IA^b mice due to deletion on IA^b plus an unknown self-peptide(s) and in CL2$^{-/-}$ mice because of the lack of a positively selecting ligand. However, they did develop into mature CD4$^+$ single-positive cells in IA^b-SP (in this case, IA^b-Eα) mice because, for the 20.4 TCR, this ligand is sufficient for positive, but not negative, selection (Figure 6C). When on a positively selecting background, mature CD4$^+$ T cells in all three of these transgenic mice accumulated in peripheral lymphoid organs as expected and responded to IA^b + 3K (Figures S4 and S6).

The patterns of development for the last two TCRs were not so straightforward. As expected, T cells bearing the other TCR derived from IA^b-SP mice (62.8 TCR-Tg derived from the YAe5 62.8 T cell hybridoma) were also deleted before maturing to the single-positive stage in wild-type IA^b-expressing thymuses but developed into mature CD4$^+$ cells in a IA^b-SP thymus (Figure 6D). However, in CL2$^{-/-}$ mice, mature CD8$^+$ cells appeared in the thymus. No mature T cells appeared in the thymus of mice lacking MHC class I and MHC class II. These results indicate that, in the absence of MHC class II, 62.8 TCR-Tg thymocytes can mature by positive selection on an MHC class I ligand.

A similar result was seen with 3K-36 TCR-transgenic T cells. The 3K-36 T cell was originally identified as a rare IA^b + 3K-reactive T cell present in CL2$^{-/-}$ mice. The 3K-36 transgenic T cells fail to mature in wild-type IA^b-expressing mice (Figure 6E). This appears to be due to negative selection on IA^b + a self-peptide(s). However, in CL2$^{-/-}$ mice, the T cells mature to CD8$^+$ T cells. Again, the fact that no development takes place in mice lacking MHC class I and MHC class II indicates that, in the absence of MHC class II-mediated negative selection, these cells can be positively selected on MHC class I and mature to CD8$^+$ T cells. For both the 62.8 and 3K-36 TCR-Tg mice on a CL2$^{-/-}$ background, mature CD8$^+$ T cells can be found in the peripheral lymphoid organs, and they respond to IA^b + 3K despite the lack of CD4 (Figures S5 and S6).

To support the conclusion that T cells bearing these two TCRs can be positively selected on MHC class I, we identified MHC class I + peptide ligands for the resulting mature CD8$^+$ T cells. In the case of 3K-36, we used the technique of positional scanning to screen a nonamer peptide library for peptides able to activate CD8$^+$ spleen cells from the transgenic mice, using H-2^b MHC class I-bearing, CL2$^{-/-}$ cells as presenters (Pinilla et al., 1992). We found multiple possibilities for amino acids at each position (Figure S7). To deconvolute this information, we screened a second set of synthesized peptides containing various combinations of the potential amino acids. More than half of these peptides showed at least some stimulation (Figure S8). Some of these were resynthesized, purified, and retested. Results with the two best peptide mimotopes are shown in Figure 6F. CL2$^{-/-}$ spleen cells incubated with a control peptide (3K) did not stimulate 3K-36-bearing CD8$^+$ T cells, but, when incubated with both mimotopes from the library, they did so in a dose dependent manner. Furthermore, when 3T3 fibroblasts transfected with either D^b or K^b were pulsed with one the of mimotopes and used as targets for activated CD8$^+$ T cells from 3K-36 mice, killing occurred only with K^b (Figure 6G).

MHC class I ligands for 62.8 CD8$^+$ T cells were identified more indirectly. We failed to identify any peptide mimotopes directly using the positional-scanning library, but the two mimotopes identified with 3K-36 T cells crossreacted weakly with 62.8 CD8$^+$ T cells (data not shown). Furthermore, when 62.8 CD8$^+$ T cells were tested for cytotoxicity with MHC class II-negative targets bearing H-2^b, H-2^k, or H-2^q MHC class I, the H-2^k, but not the H-2^b and H-2^q, cells were killed (Figure 6H).

Taken all together, these results show that T cells bearing these latter two TCRs can be selected by and are reactive to either MHC class I or class II.

Discussion

The TCR repertoire is randomly generated from variable germline elements and then shaped by selection in the thymus into a collection of TCRs that is specific for foreign peptides presented by the MHC alleles of the host. The role of positive and negative selection in this process has been studied for many years, but there are still unanswered questions: To what extent is the propensity of TCRs to recognize MHC bound antigens already determined in the germline versus dependent on selection in the thymus? How important is the peptide specificity of a TCR in positive selection? Does positive selection require MHC allele- and class-specific interactions with the TCR? What are the characteristics of the positively selected repertoire that is removed by negative selection? The data we have presented here offer some insight into these questions.

The Role of Negative Selection in Conferring Peptide and MHC Specificity on the TCR Repertoire

We compared T cells, reactive to IA^b plus the peptide 3K, that had come from two types of mice: those in which positive and negative selection were normal and those in which negative selection was limited. The T cells from mice with normal negative selection were very peptide and MHC specific and showed very little allo-MHC reactivity. On the other hand, many of the T cells from mice with limited negative selection were peptide promiscuous, less sensitive to mutations in the MHC α helices, and very allo-MHC reactive. Some had all three of these properties. Finally, 2 of the 3 TCRs

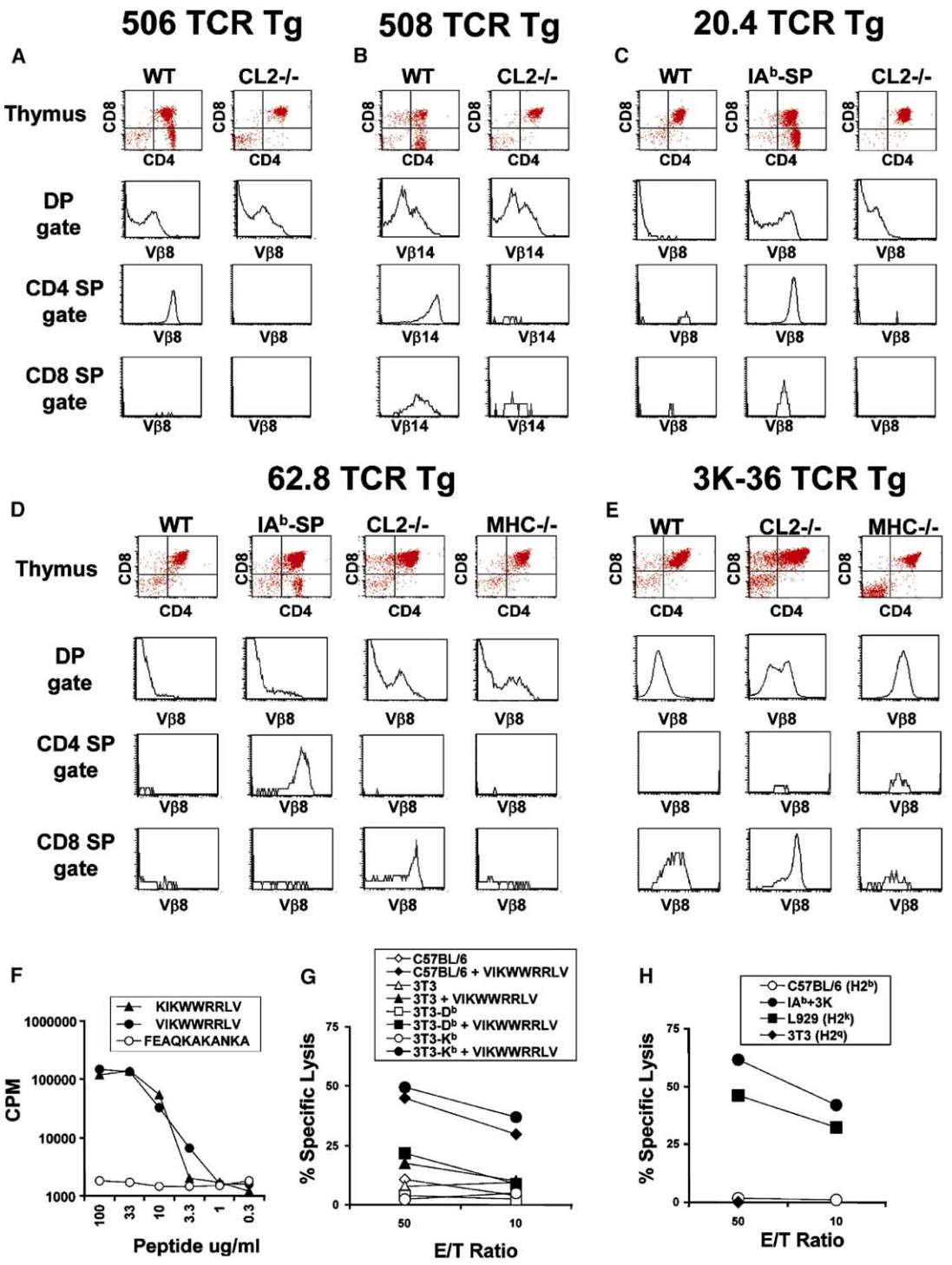


Figure 6. Thymocytes Bearing Unconventional TCRs Are Selected and Activated on MHC Class I and Class II Proteins

Thymuses from 6-week-old (A) 506, (B) 508, (C) 20.4, (D) 62.8, and (E) 3K-36 TCR-Tg Rag1$^{-/-}$ mice expressing wild-type H2^b, H2^b CL2$^{-/-}$, IA^b covalently bound to Eα (IA^b-SP), or neither class I nor class II (MHC$^{-/-}$) were stained for expression of CD4 and CD8 and Vβ expressed by the relevant TCR. Each set of four vertical panels shows CD4 versus CD8 staining for thymocytes from the indicated TCR-Tg on the indicated MHC background followed by Vβ expression on the immature CD4$^+$CD8$^+$ double-positive or mature CD4 or CD8 T cells in the thymus. CD8$^+$ spleen cells isolated from (F) 3K-36 TCR-Tg mice were activated with MHC CL2$^{-/-}$ splenocytes pulsed with varying doses of two MHC class I peptides identified by positional scanning but not the MHC class II binding 3K peptide.

(G) Activated CD8$^+$ 3K-36 TCR-Tg T cells specifically lyse H2-K^b-expressing targets pulsed with 100 μg/ml of the VIKWWRRLV peptide but not H2^q or H2^q + H2-D^b-expressing targets pulsed with the peptide.

(H) Activated CD8$^+$ 62.8 TCR-Tg T cells lyse IA^b-3K-expressing targets as well as H2^k MHC class I-expressing L929 cells but not syngeneic CL2$^{-/-}$ C57BL/6 targets or H2^q MHC class I-expressing 3T3 cells. Data are representative of three independent experiments.

from these animals that were examined in detail reacted with peptides bound to either MHC class I or class II, a property previously shown to be rare among T cells from normal mice (Wise et al., 1999).

Restoration of normal negative selection to the IA^b-SP TCR repertoire resulted in loss of the very MHC- and peptide-crossreactive T cells, leaving T cells with peptide and MHC specificities very similar to those of normal mice. The implication of these results is that germline-encoded TCR elements + CDR3 variations create a spectrum of TCRs. At one end are those quite specific for many of the amino acid side chains of their MHC + peptide ligands, and at the other end are those that recognize general conserved features of MHC + peptide molecules rather than specific amino acid side chains. Thus, the experiments presented here indicate that the positively selected TCRs can be much more broadly MHC reactive and peptide promiscuous than previously appreciated and that it is negative selection that culls this population to produce a T cell repertoire specific both for peptide and host MHC alleles.

The Role of Positive Selection in Conferring MHC-Allele Specificity on the TCR Repertoire

The conclusion that negative selection drives the T cell repertoire toward specific recognition of peptide and MHC allele raises the question of the function of positive selection in TCR-repertoire development. The original experiments demonstrating positive selection showed that T cell development results in a repertoire that is MHC-allele specific (Bevan, 1977; Scott et al., 1989). Though initially controversial (Matzinger, 1993), these results are often interpreted to mean that positive selection is driven by contact of the TCR with allele-specific amino acid side chains of the peptide and MHC molecule, followed by selection of a coreceptor appropriate for the specificity of the TCR (Goldrath and Bevan, 1999; Starr et al., 2002; von Boehmer et al., 2003). Our finding, that under conditions of limiting negative selection, many peptide-reactive T cells develop that are not MHC-allele specific, suggests an alternate role for positive selection. We propose that positive selection functions to select TCRs throughout a spectrum of specificity for MHC-conserved and allele-specific features. Self-tolerance then biases the TCR repertoire away from conserved interactions to interactions with unique side chains of the peptide and both MHC α helices, some allele specific and class specific.

The Basis of TCR Reactivity with MHC

The idea that receptors on lymphocytes might be intrinsically biased toward reaction with MHC proteins was first proposed more than 30 years ago (Jerne, 1971). It has been difficult to tackle this question experimentally, but there are some relevant data. We previously showed that random combinations of TCR α and β chains were surprisingly likely to react with MHC (Blackman et al., 1986). When two groups examined the MHC reactivity on thymocytes that had not been subjected to positive or negative selection, they found that 5%–10% of preselection TCRs reacted with MHC, a frequency perhaps much higher than would be expected from random receptor sequences (Merkenschlager et al., 1997; Zerrahn et al., 1997). Therefore, it seems likely that a sizable proportion of the random repertoire is MHC reactive. Consistent with previous findings, our results suggest that many of these MHC-reactive cells are capable of being positively selected but that very few survive negative selection (Mathis and Benoist, 2004).

When the solved structures of TCRs bound to MHC-peptide ligands began to appear, it was hoped that the evolutionarily conserved general rules that predispose the TCR to interact with MHC ligands would be revealed. However, while the alignment of TCRs on their MHC-peptide ligands is loosely similar from one combination to another, the expected conserved interactions between the TCRs and MHC have not yet been seen (Housset and Malissen, 2003).

This could be because TCRs are like immunoglobulins, with germline-encoded random specificities. The obsession of TCRs for MHC would then be controlled entirely by positive selection. The results presented here, however, suggest a different explanation: that almost all the thymocytes bearing TCRs that would illustrate the evolutionarily conserved general rules of TCR-MHC interaction are deleted by negative selection in the thymus. The TCRs that would be most useful to demonstrate the rules can only be found, as we show here, in animals in which negative selection is limited.

The same set of germline variable elements are used by TCRs to recognize MHC class I and class II, although there are a few cases in which particular variable elements are more likely to be found in T cells recognizing a particular MHC class (MacDonald et al., 1988; Sim et al., 1996). In normal mice, however, almost all TCRs react with only class I or class II, not both. Our studies here, however, revealed that at least 2 of the 3 crossreactive TCRs, which had been subject to limited negative selection, could recognize peptides associated with both class I and class II. Below, we suggest two possible, nonexclusive ways in which this might be achieved.

The Basis of TCR Reactivity with MHC: Similarities between the Amino Acid Sequence of Class I and Class II Proteins

Though it has been appreciated for some time that the overall geometry of all MHC proteins as well as the position of the bound peptide is quite similar (Brown et al., 1993), the molecular basis of MHC-class discrimination has not been elucidated. The two TCRs studied in detail here were crossreactive with peptides presented by IA^b and K^b, the structures of which have been solved (Fremont et al., 1992; Liu et al., 2002). Figure 7A shows the α helices and bound peptides of these two molecules superimposed in the conventional way to maximize backbone identity. The similarity between the upwardly pointing side chains of amino acids on the α helix of the IA^b β chain and those of the αII domain of K^b is striking. In addition, the central upwardly pointing amino acid of the peptide bound to IA^b (P5) superimposes well on the central upwardly pointing amino acid of peptide bound to K^b (P4). On the other hand, in this alignment, there is little similarity between the upwardly pointing amino acids of the IA^b α chain and the corre-

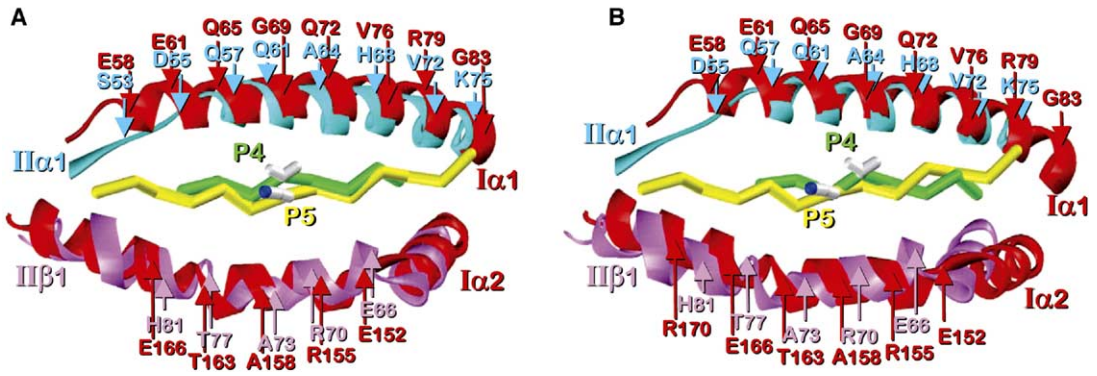


Figure 7. Structural Comparison of IA^b + 3K with K^b Presenting a Peptide from Sendai Virus Reveals Extensive Amino Acid Homology at Potential TCR Contact Residues

(A) A conventional overlay of IA^b (Liu et al., 2002) and K^b (Fremont et al., 1992) shows extensive amino acid homology at potential TCR contact residues of the IA^b β chain (magenta) and the K^b αII domain (red) including the overlap of the peptide's central up residue of K^b with IA^b.

(B) Shifting the alignment by a single turn of the α helix of the IA^b versus K^b reveals extensive homology between the $IA^b\alpha$ (cyan) and the αI domain of K^b.

sponding amino acids of the αI domain of K^b. However, if the α helix of the IA^b α chain is shifted by one turn, homology between the IA^b α chain and the αI domain of K^b becomes quite apparent (Figure 7B). Similar results are obtained when comparing the sequence of other alleles of MHC class I and class II (Figure S9).

Both of the MHC-class-crossreactive TCRs studied here were "α centric" in their interactions with IA^b, i.e., they were more sensitive to mutations in the amino acids on the top of the α helix of the IA^b α chain than the IA^b β chain. This predicts that these TCRs might react with MHC class I in the second "shifted" configuration (Figure 7B). This would allow these TCRs to engage similar amino acids of the IA^b α chain and the αI domain of K^b. Conversely, we could predict that the "β centric" TCRs from our collection may, if they are crossreactive with MHC class I, react with amino acids that are similar between the K^b αII domain and $IA^b\beta$ α helix, thus binding class I and class II in their conventional alignments (Figure 7A). TCRs that have been through normal negative selection require contacts on both MHC α helices (see Results). To bind similar residues on MHC class I and class II, they would have to shift their register by one turn on one α helix versus the other, probably a difficult feat while staying peptide reactive. This potential shift, along with negative selection's push toward dependency upon allele-specific side chains, may account for the rarity of MHC-class-crossreactive T cells in the normal repertoire.

The Basis of TCR Reactivity with MHC: Interaction of TCRs with the Backbone of MHC Proteins

The data here show that TCRs can be found that are relatively insensitive to changes in the amino acids of the peptide and on the tops of the MHC α helices. These TCRs suggest that crossreactivity between class I and class II, and overall MHC reactivity, may not be entirely due to reaction between parts of the TCRs and specific side chains of the MHC proteins, as argued above. Instead, the TCRs that are very crossreactive may react with another element that is conserved between class I and class II, the backbone of the proteins.

To explore this idea, we examined ten published TCR-MHC-peptide structures to measure the percentage of TCR interactions that are with backbone atoms of the MHC. The analysis revealed that between 22% and 46% of all hydrophilic and hydrophobic interactions between the TCRs and their ligands involved reaction of TCR residues with backbone atoms of the MHC (Figure S10). Thus, interactions between TCRs and the backbone of MHC are frequent and may prove to be the basis for the very crossreactive TCRs that we observe when negative selection is limited. These interactions may have been conserved during the coevolution of TCRs and MHC, to bias TCRs toward reaction with MHC rather than other proteins.

How could evolution select for TCR segments with affinity for generic features of MHC proteins if all the TCRs that illustrate this point disappear in the thymus, before they could be of use for the survival of their host? We believe that the explanation lies in the combinatorial properties of TCR genes. Any given TCR is produced by combining a chosen Vα, Jα, Vβ, Dβ, and Jβ segment together with junctional variations in the CDR3 regions (Davis and Bjorkman, 1988). Evolution may select for Vαs and Vβs with, for example, low but appreciable ability to bind generic features of MHC proteins. In any combination of TCR segments plus CDR3 variants, this ability may be more or less manifest, depending on the component parts. The drive to self-tolerance and MHC restriction is then accomplished by shifting this random TCR repertoire to one dependent on amino acid side chains of the peptide and MHC allele. The TCRs that survive positive and negative selection may display just portions of the conserved interactions, sufficient to allow them to have the ability to engage self-MHC but not enough to allow them to be negatively selected. Thus, the selected, useful repertoire of TCRs

in any given animal is stamped with the faint imprint of what has been selected over time in the species.

Experimental Procedures

Mice

C57BL/6, β2-microglobulin ($\beta2m^{-/-}$), and $Rag1^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, Maine). $IA^b\beta$ ($CL2^{-/-}$) mice were from Taconic. $CL2^{-/-}$, Ii-deficient ($Ii^{-/-}$), IA^b-Eα, and IA^b-2W1S single-peptide transgenic mice (IA^b-SP) have been previously described (Ignatowicz et al., 1996; Huseby et al., 2003). TCR-Tg mice were established by expressing rearranged PCR-cloned TCRs (Huseby et al., 2001a) using the human CD2 promoter (Zhumabekov et al., 1995) (see Supplemental Data). All mice were maintained in a pathogen-free environment in accordance with institutional guidelines in the Animal Care Facility at the National Jewish Medical and Research Center.

Immunization of Mice and Generation of T Cell Hybridomas

Mice were immunized with DC expressing IA^b-3K, and responding T cells were isolated and converted into hybridomas as previously described (Huseby et al., 2003). T cell hybridomas are named B3K if from C57BL/6 mice, 72- and 73- if from IA^b-Eα SP mice reconstituted with C57BL/6 BM, YAe if from IA^b-Eα SP mice, 2W1S if from IA^b-2W1S SP mice, or 74- and 75- if from IA^b-Eα SP mice reconstituted with IA^b-Eα BM. Some of the IA^b + 3K-specific T cell hybridomas have previously been described (Huseby et al., 2003).

Hybridoma and T Cell Stimulation with APC Expressing Amino Acid Substitutions in IA^b + 3K

A fibroblast cell line was used as the recipient cell for a series of MSCV-based retroviruses expressing the cDNAs for $IA^b\alpha$ and 3K-variant-peptide-linked $IA^b\beta$ (Huseby et al., 2003) (Figure S1). To generate the MHC mutations, an insect-cell-surface-displayed version of IA^b with covalently coupled 3K peptide was expressed using baculovirus and expressed on SF9 cells transfected with B7.1 and ICAM-1 (Crawford et al., 2004).

10^5 T hybridoma cells per well were incubated with 3×10^4 of each of the IA^b-SP fibroblasts, B7.1/ICAM-1 SF9 cells expressing IA^b + 3K mutations, or 10^6 spleen cells expressing various MHC alleles for 24 hr, and their supernatants were screened for IL-2 content (Huseby et al., 2003). For Tg T cell stimulation assays, 10^5 Tg T cells were incubated in 200 μl S-MEM with 3×10^4 irradiated (7000 rad) IA^b-SP-presenting cells or 5×10^5 peptide-pulsed mitomycin C-treated $CL2^{-/-}$ spleen cells for 48 hr in flat-bottom 96-well plates followed by a 12 hr pulse of [^{3}H]thymidine.

Cytotoxicity experiments were done by activating T cells with 2×10^7 spleen cells from the various TCR-Tg mice with 3×10^7 mitomycin C-treated C57BL/6 spleen cells preincubated with 0.1 μg/ml 3K peptide in 10 ml cultures for 6 days. Cytotoxicity assays were done using standard techniques (Huseby et al., 2001b).

MHC-Peptide Tetramers and Soluble TCR Proteins

Soluble IA^b + 3K and TCRs were produced and purified as described (Crawford et al., 2004; Liu et al., 2002). IA^b + 3K tetramers were produced as previously described (Crawford et al., 1998). T cell hybridomas were stained with either the anti-TCR Cβ antibody HAM57-597 or IA^b + 3K tetramers at 37°C for 2 hr (Crawford et al., 1998). Background tetramer staining intensity was determined by staining an IA^b-restricted T cell specific for the Eα peptide. The relative affinities of the TCRs were then determined by dividing the specific tetramer staining by the total TCR staining intensity (tetramer staining – background)/(total TCR staining – background). Because the background relative affinity (no specific tetramer staining) for the negative control TCR was 0.01, we set this as the negative value.

Statistical Analyses

Mann-Whitney tests were done to compare the crossreactivities of T cells tolerized on C57BL/6 versus T cells tolerized by IA^b bound to a SP. Comparisons of analyses of peptide recognition included responses to all 95 amino acid substitutions. For analyses of the crossreactivities of the two types of TCR with the entire MHC-peptide surface, each of the 15 MHC + peptide-substituted positions were given a score of 2, 1, or 0 depending on their ability to tolerate >74%, 26%–74%, or 25% or less substitutions. Both comparisons showed that the population of TCRs tolerized on IA^b-SP was significantly more crossreactive than that tolerized on B6 (see Results).

One-tailed Student's t tests were used to compare the reactivities of individual TCRs from IA^b-SP mice to those of the entire populations of TCRs tolerized on C57BL/6. Using this method with a $p < 0.05$ confidence level, multiple TCRs were less sensitive to mutations of the $IA^b\alpha$, $IA^b\beta$, or the entire MHC-peptide surface (see Results).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and ten figures and can be found with this article online at http://www.cell.com/cgi/content/full/122/2/247/DC1/.

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