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Thymic selection stifles TCR reactivity with the main chain structure of MHC and forces interactions with the peptide side chains

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The stem cells which seed the thymus bear neither receptors for antigen nor the coreceptors which are characteristic of mature T cells, CD4 or CD8. After entering the thymus, cells destined to bear T cell receptors (TCRs) made up of α and β chains, construct the genes for these chains by rearranging one of many V α s to lie next to one of many J α s, and one of many V β s to lie next to one or more version of D β and one of a number of J β s (Jackson and Krangel, 2006). Since all these elements are encoded by the germ line, one might imagine that TCR+ thymocytes might have a very large, but genetically fixed, set of specificities for antigen. However, nucleotides can be removed or introduced at the junctions between these elements, and thus these joining points (N regions) are quite random, and not genetically controlled (Davis and Bjorkman, 1988). N regions code for part of the CDR3 loops of TCR α and β polypeptides and the CDR3 regions are a major component of the area of TCRs which contact their ligands (Rudolph et al., 2006). Consequently the germ line specificities of TCRs are modified by their nongerm line N regions and thymocytes bear TCRs which manifest their germline encoded specificities to varying degrees.

$\alpha\beta$ TCRs are thought to be quite MHC and peptide specific

Not all thymocytes are destined to become mature T cells. Selection in the thymus tests the specificities of the TCRs on each thymocyte and, based on these, picks out which cells are allowed to mature. Many experiments have demonstrated that thymocytes expressing TCRs with little or no affinity for self peptides bound to host Major Histocompatibility Complex (MHC) proteins die at this stage due to the failure to receive a survival signal through their TCR. Developing thymocytes expressing a TCR with a relatively high affinity for self peptides bound to host MHC proteins undergo negative selection and also die, however this is a TCR signaled event. Only thymocytes which express a TCR with a “moderate affinity” for self peptides bound to host MHC proteins are signaled to live, a process called positive selection (Goldrath and Bevan, 1999; von Boehmer et al., 2003). These thymocytes, then have the choice to become either a CD4⁺ T cell or a CD8⁺ T cell.

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A plethora of studies have suggested that although positive selection requires only moderate affinity of TCRs for MHC + peptide, the event is quite specific. Often, a given TCR requires a unique set of peptides bound to particular allele of MHC class I or MHC class II proteins to permit positive selection (Berg et al., 1989; Scott et al., 1989; Bevan, 1997). Similarly, studies of peripheral T cell responses have shown that TCRs on mature T cells are relatively peptide and MHC class and allele specific. That is, only a subset of peptides, bound to specific class and alleles of MHC, are able to activate each T cell (Zinkernagel and Doherty, 1974; Babbitt et al., 1985; Townsend and Bodmer, 1989). Because of these experiments, the dominant model of TCR recognition of ligand has been that TCRs are inherently peptide and MHC class and allele specific. There is however a major caveat to this hypothesis, which is that the experiments that suggest thymic selection requires peptide and MHC class and allele specific interactions have all analyzed TCRs which were isolated from mature T cells. Thus, these experiments are studying the development and specificity of TCRs that have already been skewed by the pressures of thymic selection. It is possible that the preselected repertoire of TCRs includes receptors which have different properties.

Preselected TCRs may include some which are very likely to react with any MHC protein

Selection in the thymus generates a collection of mature T cells which are very likely to react with self MHC bound to some peptide or another. About 1–5% of the TCRs on these cells have the ability to react also with foreign MHC proteins, bound to the collection of peptides which these proteins are engaged in normal animals (Wilson and Nowell, 1970; Suchin et al., 2001). A few experiments have measured the ability of the TCRs on thymocytes before selection to react with self or foreign MHC and these suggest that the preselection repertoire includes TCRs which are more likely to be crossreactive than predicted by studies of mature T cells. During thymic selection, only 1–2% of thymocytes are allowed to develop and become mature T cells (Huesmann et al., 1991). However, when pre-selection thymocytes were tested for their ability to interact with MHC proteins presenting endogenous peptides, depending upon the number of MHC alleles present, 6–20% of all thymocytes recognized these MHC + peptide complexes (Yeh et al., 1984; Merckenschlager et al., 1997). Additionally, when CD4⁺ T cells from mice which didn't undergo positive or negative selection were tested for MHC + endogenous peptide reactivity, 6–15% of these T cells were reactive to endogenous peptides bound to some allele of MHC (Zerrahn et al., 1997; Henderson et al., 1998). Because the expression of the T cell co-receptor CD8 is generally required for class I reactive T cells to recognize peptides bound to MHC class I proteins and the mature T cells that were tested for alloreactivity bore only the CD4 co-receptor, these MHC reactivity frequencies are likely to be an underestimate (Potter et al., 1989; Fung-Leung et al., 1991).

In another approach to the same problem, we studied the specificities of random pairs of TCR α and β chains and found that such pairs often reacted with MHC. The frequency with which this happened was much higher than would have been expected, had the specificities of TCR chains been completely random (Blackman et al., 1986).

Finally, a clue to the specificities of the entire TCR repertoire comes from the frequency with which thymocytes are allowed to mature. Estimates vary, but on the whole it is thought that only about 1% of all these cells turn into mature T cells. Why do the other 99% fail? It may be that almost all of these cells bear TCRs which are unable to react with self MHC, thus failing because they do not pass the test of positive selection. On the other hand, some of these cells might fail because their TCRs react too well with self MHC, and the cells therefore die by negative selection. Several experiments have tried to measure the proportion of thymocytes which are culled by either process, using mice in which negative selection is limited in some way. For example, the CD4⁺ T cells that develop in mice that contain only one class II MHC

protein, IAb, bound to a single peptide (IAb-SP mice) have a frequency of self- and allo-MHC + peptide reactivity that is far higher than that of T cells in mice with normal negative selection (Fung-Leung et al., 1996; Ignatowicz et al., 1996; Laufer et al., 1996; Miyazaki et al., 1996; Fukui et al., 1997). Overall, these data suggest randomly generated TCRs are somehow biased towards MHC + peptide reactivity and that negative selection has a role in getting rid of the TCRs which manifest this too well.

The hypotheses: The germ line repertoire of TCRs is evolutionarily selected to react with the main chain and invariant amino acids of MHC. Because of tolerance, TCRs which manifest this property appear only rarely on mature T cells

Although the obsession of TCRs on mature T cells for MHC can be explained by a well established phenomenon, positive selection in the thymus, the rate of MHC reactivity in the pre-selection repertoire suggests this can't be the only solution. To account for the MHC bias of the random, preselection TCR repertoire we have generated and started testing a modern version of a hypothesis put forward 35 years ago by Niels Jerne (Jerne, 1971). Jerne suggested that lymphocyte receptors have been evolutionarily selected to interact with MHC proteins. The modern version applies this idea to $\alpha\beta$ TCRs and not the receptors on B cells or $\gamma\delta$ T cells. The hypothesis is guided by the following observations.

1. The germ line encoded portions of the TCR which contact MHC, V α , J α , V β , D β and J β , are used in TCRs which can react with either MHC class I or MHC class II proteins. Although there is some preference for certain TCR elements to react with one or the other type of MHC, this preference is not absolute (Sim et al., 1996). Therefore, if there is evolutionary selection for TCR elements to react with MHC, the selection must not distinguish between binding to the 2 classes of MHC.
2. MHC genes are among the most polymorphic of the species with man having > 800 alleles of MHC class I and > 600 alleles of MHC class II (Robinson et al., 2003). TCR genes, on the other hand, although many, do not vary very much between different members of the species and there is no evidence that the few polymorphisms of TCR genes which have been studied correlate with polymorphisms at MHC. Therefore, if TCR elements have been selected evolutionarily to react with MHC, they must be able to cope with all the polymorphic variants of MHC expressed in the species.

To accommodate these issues, we hypothesize that the germ line encoded TCR gene segments are biased towards recognition of the only features of MHC which are common to class I and class II, and to any allele of these proteins in the species. These features are the main chain structure of the proteins and the few invariant amino acid side chains on the face of the proteins which reacts with TCRs.

We suggest that different combinations of V α N α J α and V β D β N β and J β will give rise to TCRs which, to different degrees, manifest these specificities. Positive selection will pick out thymocytes bearing TCRs that pass a certain threshold of affinity for MHC + self peptides. Since the germ line TCR repertoire is biased towards recognition of MHC, positively selected thymocytes will often have a generic ability to react with MHC. However, regardless of the MHC alleles expressed, almost all of the cells expressing these generically MHC-reactive TCRs will die in the thymuses of any individual of the species. The MHC cross reactivity of the TCRs will determine this fate. Thus the generically cross reactive TCRs will never appear on mature T cells. This idea is illustrated in Figure 1.

Moreover, negative selection makes the repertoire of TCRs on mature T cells relatively specific for MHC and for peptide, by removing the cells which bear very cross reactive TCRs.

Tests of the hypotheses

TCRs which have been through minimal negative selection are relatively peptide and MHC cross reactive

To test our hypothesis that TCRs are biased towards recognition of conserved features of MHC, we asked two questions. 1) For TCRs reactive to a particular MHC + peptide complex, how broad is the range of peptide and MHC specificity? 2) If there is a significant range of MHC + peptide specificity, does negative selection affect it?

To address these questions, we compared the properties of the TCRs on T cells from normal mice with those on T cells from a repertoire that was less reduced by negative selection (Huseby et al., 2005). We chose to use IA^b-SP mice as a source of T cells which have not been skewed by conventional negative selection. In these mice, negative selection occurs on IA^b bound to only one peptide (the E α peptide) and not on IA^b + the many thousands of self peptides normally present within the thymus (Ignatowicz et al., 1996). To ensure that any phenotype we might find was due to the lack of negative selection and not to positive selection on the IA^b-SP complex, we constructed bone marrow (BM) chimeric mice in which we reconstituted IA^b-SP mice with BM from C57BL/6 (B6) mice. In these mice, positive selection occurs on the IA^b-SP while the BM-derived APC from the C57BL/6 mice ensure that proper negative selection occurs. Thus, we had three types of mice: mice with a TCR repertoire in which negative selection had been drastically diminished (IA^b-SP mice), mice with a conventional TCR repertoire (C57BL/6 mice), and mice containing T cells that have gone through limited positive, but normal negative, selection (chimeric mice) (Huseby et al., 2005).

These mice were immunized with dendritic cells presenting IA^b + the 3K peptide. The 3K peptide is a variant of the E α peptide in which contains a G to K substitution at P3, a L to K substitution at P5 and an I to K substitution at the P8 position. All of these substitutions are at positions of the peptide that point out from the MHC binding cleft and hence are potential TCR contact residues. The responding IA^b+3K reactive T cells from these mice were converted to T cell hybridomas and their peptide and MHC specificities were evaluated.

To measure peptide specificity, we tested the ability of the T cell hybridomas from the various mice to be activated by a series of cell lines that expressed variants of the 3K peptide in which each of the 5 potential TCR contact positions were mutated to the other 19 possible amino acids. In IA^b-SP mice, a set of T cells (12/30 tested) were significantly more peptide degenerate than T cells isolated from C57BL/6 mice. Whereas T cells from C57BL/6 mice always recognized specifically 3 or more amino acid side chains of the 3K peptide, some T cells from IA^b-SP mice only recognized specifically one side chain. For these extremely peptide degenerate TCRs, the one required position was usually the central P5 position. The slightly less degenerate TCRs, which recognized 2 positions of the peptide, were almost always specific for the P8 position of 3K paired with either P-1, P2, P3 or P5. In addition to these peptide degenerate TCRs, IA^b-SP mice also contained T cells which were quite peptide specific, like those from the negative selection sufficient C57BL/6 mice. Interestingly, all of the T cells from chimeric mice were highly peptide specific (Huseby et al., 2005). Thus, as was found in recent studies of alloreactive T cells, the fundamental repertoire of TCRs contains receptors with varying specificities for peptide and negative selection removes TCRs with degenerate specificities from the mature repertoire (Sherman and Chattopadhyay, 1993; Daniel et al., 1998; Obst et al., 2000; Huseby et al., 2003).

We guessed that the degenerate TCRs must not be getting much binding energy by reacting with the peptide, therefore we expected that these TCRs might be more specific than normal TCRs for the side chains of the MHC. We tested this idea by measuring the ability of the TCRs in our collection to react with IA^b, mutated in each of its residues which point towards the TCR, bound to 3K. We were surprised to find that some of the TCRs which reacted with many versions of 3K also reacted with many versions of IA^b. All these cross reactive TCRs came from the IA^b-SP mice. TCRs from C57BL/6 or chimeric mice were all relatively specific for the side chains of IA^b (Huseby et al., 2005).

The crossreactive TCRs have normal kinetics of binding to their ligands

Recent experiments have shown that the specificity of a TCR is maintained even when it is mutated to be of higher and higher affinity for its ligand, suggesting that exquisite specificity is not necessarily related to affinity (Weber et al., 2005). Likewise, we find that the highest affinity TCRs are not always the most degenerate TCRs. Surface plasma resonance experiments show that the MHC + peptide degenerate TCRs often had binding kinetics and equilibrium binding affinities which are similar to those of MHC + peptide specific TCRs. Thus the cross reactive properties of the degenerate TCRs are not caused by odd on and off rates or peculiar affinities for their MHC/peptide ligands (Huseby et al., 2005).

The cross reactive TCRs react with both class I and class II

To follow the development of the MHC + peptide specific and degenerate TCRs in vivo, we created a series of TCR Tg mice. We were unsure how the very cross reactive TCRs would behave during thymic development. Interestingly, we found the MHC + peptide degenerate YAe5 62.8 TCR underwent positive selection on both the IA^b-SP as well as H2^b MHC class I proteins. The ability of this TCR to undergo positive selection on MHC class I proteins was not a function of specificity for an odd peptide, 3K, since 2 other TCRs derived from conventional C57BL/6 mice that were also specific for the IA^b+3K ligand required IA^b for positive selection. The YAe5 62.8 TCR is not a one-of-a-kind TCR as the IA^b+3K reactive 3K-36 TCR, isolated from a MHC class II deficient mouse, was also positively selected on both MHC class I and MHC class II proteins (Huseby et al., 2005).

To make sure that these IA^b+3K reactive, MHC class cross reactive TCRs were really specific for MHC class I proteins + peptides, we screened a combinatorial peptide library to identify peptide antigens that could activate CD8⁺ T cells expressing these TCRs. We were able to identify peptide antigens for both the YAe5 62.8 and 3K-36 that, when bound to H2-K^b, could activate the corresponding CD8⁺ T cell (Huseby et al., 2005) (and unpublished data). In addition, the YAe5 62.8 TCR, it was found to be alloreactive with H2^k MHC class I proteins. The two MHC class cross reactive TCRs we have studied in great detail are not the only TCRs that are able to simultaneously recognize peptides bound to MHC class I and class II proteins. There is a growing list of TCR which have this capability, whether they be responses to allogeneic MHC class mismatched ligands or arise naturally in a host (Schilham et al., 1986; Aparicio et al., 1987; Arsov and Vukmanovic, 1999; Heemskerk et al., 2001; Morgan et al., 2004; Serreze et al., 2004; Huseby et al., 2005; Logunova et al., 2005; Eshima et al., 2006; Ge et al., 2006). Because many of these MHC class cross reactive TCRs are also autoreactive, it will be of utmost interest to decipher how these TCRs are recognizing their ligands and if this class cross reactivity is a general property of the more degenerate and autoreactive TCRs that escape negative selection.

TCRs may focus on one helix of the MHC protein

Some of the TCRs from the IA^b-SP mice had another property of interest, they tended to react specifically with amino acids on only one of the IA^b alpha helices. Thus, some of these TCRs

were biased towards reaction with specific side chains of the $IA^b\alpha$ chain but not the peptide or $IA^b\beta$ chain, and others towards reaction with specific side chains of the $IA^b\beta$ chain but not the peptide or $IA^b\alpha$ chain (Huseby et al., 2005). The $IA^b\alpha$ chain specific TCRs are reminiscent of an autoreactive TCR, the 172.10 TCR specific for IA^u plus the Ac1-11 peptide derived from myelin basic protein (Maynard et al., 2005). The interface of this receptor/ligand complex is dominated by the TCR $V\beta$ loops interacting with the $IA^u\alpha$ chain with very little interaction with the $IA^u\beta$ chain.

Finding T cells that were MHC class cross reactive as well as biased towards one of the α -helix of MHC, led us to re-analyze the amino acid similarities between MHC class I proteins and MHC class II proteins (Bjorkman et al., 1987; Fremont et al., 1992; Brown et al., 1993; Liu et al., 2002). In a conventional overlay many of the upwardly pointing residues of the $IA^b\beta$ chain, for example, and the $\alpha 2$ domain of $H2-K^b$, for example, are identical. However in this same alignment the α chain of IA^b and the $\alpha 1$ domain $H2-K^b$ do not share much homology. On the other hand, if the α -helix of IA^b is shifted by one turn, extensive homology between the α chain of IA^b and the $\alpha 1$ domain of $H2-K^b$ appears (Fig. 2). In addition to homology between IA^b and $H2-K^b$, extensive amino acid homology at potential TCR contact residues can be found for all MHC class I proteins compared to MHC class II proteins. Thus, it is possible that these MHC class cross reactive TCRs are recognizing patches of homology on the different MHC proteins.

Cross reactive TCRs may have shorter CDR3 regions

The MHC and peptide degenerate T cells from IA^b -SP mice were often extremely poly-MHC reactive. For example, the YAe5-62.8 T cells were activated by 7 of 9 MHC haplotypes tested. In all, T cells from IA^b -SP mice reacted with 70/171 different MHC haplotype with which they were challenged. This is in sharp contrast to the T cells from C57BL/6 mice which had a rate of reactivity with allogeneic MHC of 2/63 (Huseby et al., 2005).

Interestingly, when the CDR3 α lengths of different T cells were plotted against their MHC reactivity, the most poly-MHC reactive TCRs often had the shortest CDR3 loops (Fig. 3). The outlier (circled in Fig. 3) has a CDR3 α which differs from the others in its high content of glycine residues, perhaps allowing this CDR3 to be more flexible than most. This suggests that the TCRs which are very cross reactive are distinguished from non-cross reactive TCRs by their CDR3 regions, not by their germ line encoded elements. The idea that long CDR3 regions interfere with MHC recognition is supported by the observation that CDR3 β lengths are shorter in positively selected versus pre-selected thymocytes (Yassai and Gorski, 2000; Yassai et al., 2002). Additionally, T cells that are generated in the absence of TdT, an enzyme that adds nucleotides at the junctions between the TCR V, D and J gene segments, are more peptide degenerate than conventional T cells with longer CDR3 loops (Gilfillan et al., 1993; Komori et al., 1993; Gavin and Bevan, 1995).

Why would the length of the CDR3 regions affect cross reactivity? Cross reactivity requires that the TCRs react with elements which are common to different MHC proteins. Long CDR3 regions will tend to lift TCRs away from the MHC, thus precluding interaction with these common elements and forcing the TCR, via its CDR3 region to interact with portions of the MHC/peptide which are not shared with other such ligands. Since the CDR3 regions are not germ line encoded, they will not have been biased by evolution towards recognition of any particular conserved feature of MHC and are thus free to focus on the portions of the ligand which are unique, thus imposing specificity on the TCR involved.

Features which are shared by all MHC proteins

We suggest (see above) that evolution has selected for TCR elements with an intrinsic ability to bind to features of MHC which are common the class I and class II proteins, and to any allele

of these proteins. A few amino acids are conserved between the many versions of MHC, and they might serve as conserved targets for binding by all or subsets of TCR V regions. Indeed, the observation that class I and class II have homologous patches of amino acids on either of their alpha helices, depending on the alignment placed on the proteins (see above) suggests that this is possible.

However, classical class I and class II proteins have another property in common, the shape of their alpha carbon backbone and the position of the amino acid beta carbons. We suggest that TCR V regions have been selected to react with this backbone. In fact, in all protein interfaces there is a balance between interaction with amino acid side chain determinants and interactions with determinants of the main chain. In a review of 10 TCR-MHC + peptide interfaces, we found that 32–57% of all contacts between the TCRs and their MHC + peptide complex are with determinants of the MHC or peptide main chain (Huseby et al., 2005). Similarly, in a recent review by Wilson and colleagues of all 23 crystal structures of TCRs bound to classical MHC + peptide complexes, only 15–53% of the contacts of the TCR were with side chains of the peptide (an example is shown in Figure 4) (Rudolph et al., 2006). Though neither the number of contacts nor the amount of buried surface between two proteins necessarily correlates with the affinity contribution of the interaction, it does suggest there is a fine balance between the energy contribution of the peptide side chains and the rest of the MHC protein (Clackson and Wells, 1995). Therefore, one possible solution for how the MHC + peptide side chain less-dependent TCRs we identified from negative selection limited IA^b-SP mice are generating their binding affinity is that they get a correspondingly greater amount from main chain determinants of the MHC + peptide complex.

Summary

We believe that evolution has selected for TCR V regions which can bind to any class or allele of MHC in the species. One target for such recognition are the conserved patches of amino acids which are shared between class I and class II, regardless of allele. Another target is the main chain of classical class I and class II proteins, since the main chain has the same configuration in all versions of these proteins.

We suggest that these evolutionarily selected targets for TCRs are not well recognized by the TCRs on mature T cells because negative selection in the thymus kills off the thymocytes bearing the TCRs which react too well with MHC.

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Negative selection forces TCRs to be dependent upon amino acid side chains of the peptide:

Fig. 1. Positive and negative selection filter the fundamental repertoire of TCRs such that the TCRs on mature T cells are relatively MHC and peptide specific

(A) The TCRs in the preselection repertoire have, to varying degrees, the ability to react with specific side chains of the peptide and conserved determinants of the MHC side chains and MHC and peptide main chain. (B) Positive selection selects all TCRs that a certain level of affinity for endogenous peptides bound to host MHC proteins. Because there are only a few thousand peptides presented by host MHC proteins, the TCRs that are extremely peptide specific and generate all of their binding affinity from the peptide are unlikely to be selected. (C) Negative selection eliminates TCRs that have a high affinity for a self peptide presented by host MHC. Negative selection also eliminates TCRs that have too much MHC + peptide cross reactivity, TCRs that generate too much binding affinity from the conserved determinants due to the immense avidity of these TCRs for all MHC proteins expressed in the host.

A. MHC Class I

B. MHC Class II

C. Conventional Overlay

D. Shifted Overlay

Fig. 2. Comparison of H2-K^b presenting a peptide from Sendai with the IA^b + 3K structure reveals a similar main chain structure as well as extensive amino acid homology at potential TCR contact residues

(A) A ribbon diagram comparison of H2-K^b main chain structure with (B) IA^b demonstrates homology of the MHC main chain structure as well as the position of the peptide (Fremont et al., 1992; Liu et al., 2002). (C) A conventional overlay of H2-K^b and IA^b shows extensive amino acid homology at potential TCR contact residues of H2-K^bαII domain (dark blue) and the IA^bβ chain (light blue) including the overlap of the peptide's central up residue of H2-K^b with IA^b. (D) Shifting the alignment by a single turn of the α-helix of H2-K^bαI domain relative to IA^b versus reveals extensive homology between the αI domain of H2-K^b (dark blue) and IA^bα (magenta).

Fig. 3. The frequency of TCR reactivity with different alleles of MHC correlates with the length of CDR3 α

(A) IA^b+3K reactive TCRs that have the highest rate of MHC reactivity have shorter CDR3 α loops than do TCRs that are less MHC reactive. The length of CDR3 α was measured by the number of amino acid residues between and including the conserved Cys at the end of the TCR V α gene segments to the conserved Phe-Gly in J α gene segments. (B) IA^b+3K reactive TCRs that have the highest rate of MHC reactivity have similar length CDR3 β loops as do TCRs that are less MHC reactive. The length of CDR3 β was measured by the number of amino acid residues between and including the conserved Cys at the end of the TCR V β gene segments to the conserved Phe-Gly in J β gene segments. The nine MHC alleles tested were H2-b, bm12, d, f, k, q, r, s and u.

TCR and MHC + peptide proteins interact via atoms of the main chains and side chain

TCR CDR2 β Tyr50 interacting with IA^k α Gln57

- A. TCR binding side chain MHC Nitrogen
- B. TCR binding main chain MHC Oxygen

Analysis of 10 TCR-MHC + peptide crystal structures

32-57% of TCR contacts are with main chain MHC atoms

Analysis of 23 TCR-MHC + peptide crystal structures

15-53% of TCR contacts are with peptide side chain atoms

Fig. 4. TCRs interact with atom of the main chain and side chains of the MHC + peptide complex (A) The TCR CDR2 β Tyr50 side chain of the D10 TCR generates a hydrogen bond with the side chain nitrogen atom of the IA^k α Gln57 residue (Reinherz et al., 1999). (B) The TCR CDR2 β Tyr50 side chain of the D10 TCR generates a hydrogen bond with a main chain oxygen atom of the IA^k α Gln57 residue.