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A New Twist In TCR Diversity Revealed By A Forbidden $\alpha\beta$ TCR

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Summary

We report crystal structures of a negatively-selected TCR that recognizes two I-A^b-restricted myelin basic protein peptides and one of its pMHC ligands. Unusual CDR structural features revealed by our analyses identify a previously unrecognized mechanism by which the highly variable CDR3 regions define ligand specificity. In addition to the pMHC contact residues contributed by CDR3, CDR3 residues buried deep within the V α /V β interface exert indirect effects on recognition by influencing the V α /V β interdomain angle. This phenomenon represents an additional mechanism for increasing the potential diversity of the TCR repertoire. Both the direct and indirect effects exerted by CDR residues can impact global TCR/MHC docking. Analysis of the available TCR structures in light of these results highlights the significance of V α /V β interdomain angle in determining specificity and indicates that TCR/pMHC interface features do not distinguish autoimmune from non-autoimmune class II-restricted TCRs.

Introduction

Numerous studies employing different experimental approaches have revealed substantial degeneracy in TCR recognition.^{1–4} Degenerate TCR recognition is required to positively select TCRs on self pMHC ligands in the thymus that are then able to recognize foreign pMHC ligands in the periphery. Multiple structures comparing bound and unbound TCRs have shown that the complementarity determining region (CDR) 3 loops, which provide many of the direct contacts to the antigenic peptide, are flexible.^{5,6} Thus, the conformational malleability of these regions may enable polyspecificity through induced-fit recognition of multiple peptides within the binding cleft of the same MHC molecule. In most previous structural studies of degenerate TCRs, however, the residues that differ between peptide epitopes are more often MHC anchor residues rather than direct TCR contacts.^{7,8} Well studied cases of degenerate recognition of totally distinct peptide/MHC complexes (pMHC) are rare.^{4,5,9,10} Here, we use the terms ‘degenerate’ and ‘polyspecific’ interchangeably to refer to the ability of one protein interface

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to specifically interact with multiple, distinct partners, an inherent property of protein recognition.

TCR degeneracy may facilitate central tolerance because a single TCR could be eliminated by multiple degenerate self-ligands rather than just one specific pMHC complex. Nevertheless, some T cells are still able to escape negative selection in the thymus. Self-reactive TCRs have been extensively studied in experimental autoimmune encephalomyelitis (EAE), a widely used animal model for multiple sclerosis. EAE is induced in B10.PL mice by immunization with myelin basic protein (MBP). Disease in these mice is mediated exclusively by CD4⁺ T cells specific for MBP_{Ac1-11}.¹¹ MBP_{Ac1-11}-specific T cells escape clonal deletion in the thymus due to poor binding of the MBP_{Ac1-11} peptide to I-A^u.¹¹⁻¹³ In addition, recent structural studies have suggested that self-reactive TCRs in the periphery escape negative selection by interacting with pMHC complexes in an atypical orientation, which may not generate a sufficiently strong interaction to induce negative selection.¹⁴ However, no structural studies have been reported for MHC class II TCRs that undergo negative selection as a comparison. T cells that are normally negatively-selected can be obtained by immunization of MBP-deficient (MBP^{-/-}) B10.PL mice with MBP. Using this approach, we identified a distinct, highly immunogenic region within MBP₁₂₁₋₁₅₁.¹³ T cells responding to this region undergo extensive thymic tolerance and are not readily detected in wild-type B10.PL mice.¹⁵ Two core epitopes identified within this region are presented by I-A^u: MBP₁₂₅₋₁₃₅ (GGRASDYKSAH) and MBP₁₃₆₋₁₄₆ (KGFGKAYDAQG).⁴ Surprisingly, many T cells isolated from MBP^{-/-} mice degenerately respond to both I-A^u/MBP₁₂₅₋₁₃₅ (pMHC₁₂₅) and I-A^u/MBP₁₃₆₋₁₄₆ (pMHC₁₃₆). Anchor and TCR contact positions for the peptides were defined by measuring both MHC binding complex half-life ($\tau_{1/2}$) and T cell responses to a series of peptide mutations.⁴ The major TCR contacts (rather than MHC anchor residues) in each peptide required for recognition by degenerate TCRs are chemically distinct, thus providing an ideal system for investigating degenerate TCR recognition of pMHCs.

Here we present the crystal structure of a TCR (1.D9.B2) bispecific for both pMHC₁₂₅ and pMHC₁₃₆, the crystal structure of pMHC₁₂₅ and a mutational scanning of the 1.D9.B2 CDR residues. This TCR was isolated from T cells obtained from MBP^{-/-} mice and has been shown to mediate negative selection in wild-type MBP^{+/+} mice.¹⁵ 1.D9.B2 (V α 2.3/V β 8.2) exhibits an unusual pattern of CDR loop flexibility, and undergoes extreme α/β interdomain movements relative to another V α 2.3/V β 8.2 I-A^u-restricted TCR due to sequence differences within CDR3. This rotation significantly alters the recognition interface of the TCR, precluding 1.D9.B2 from interacting with its I-A^u ligand in the same way seen in a previous V α 2.3/V β 8.2 TCR/I-A^u complex structure.¹⁶ Analysis of other TCR structures shows that inherent variation in the V α /V β interdomain angle is a general property of TCRs. Together, these data reveal a new molecular mechanism that expands the potential TCR specificity repertoire. In addition, we analyzed a computationally modeled 1.D9.B2/pMHC₁₂₅ complex, which mediates negative selection, in the context of all other described autoimmune and non-autoimmune TCR/MHC II complex structures. Analyses of key binding residues, determined experimentally for 1.D9.B2 and computationally for other TCR/MHC II complexes, along with detailed structural comparisons, indicated that autoimmune and non-autoimmune TCRs did not differ significantly in their interactions with pMHC complexes. These results suggest that the ability of self-reactive T cells to escape negative selection is not necessarily due to a strict focusing of binding energy on the N-terminus of the peptide.

Results

TCR/pMHC interface analysis: autoimmune versus non-autoimmune

Binding energy at protein-protein interfaces is typically unevenly distributed among contact residues, resulting in binding energy ‘hotspots’.¹⁷ Interface hotspots are not defined solely by

contact residues as non-contact residues can modulate these energies indirectly. To map the 1.D9.B2 hotspots for interaction with both pMHCs, we used site-directed mutagenesis to generate a panel of single amino acid mutations within the TCR CDR1 and CDR3 regions, which are the regions most directly involved in pMHC contacts. Mutant TCR chains were transduced into a TCR⁺ T cell hybridoma and responses to the two different MBP epitopes were compared between mutant TCR-transduced and parent TCR-transduced hybridomas. Mutations that result in loss of recognition of one but not the other MBP epitope would define residues involved in differential recognition of the pMHCs. However, all mutations either had no effect or comparably affected responses to both MBP121-140 and MBP131-150, peptides containing the two epitopes (Figure 1). Of the 27 mutants analyzed, 14 of 15 mutations made within the V α 2.3 and V β 8.2 CDR3 regions abolished responses to both MBP epitopes. In the CDR1 regions, only four of 12 mutants abolished recognition of both MBP epitopes, and the other mutations had no effect on recognition of either epitope. These data suggest that the TCR uses identical CDR1 and 3 residues to recognize both epitopes within MBP121-150.

To detect any patterns in the recognition mechanisms of class II-restricted TCRs that correlate with specific immunologic contexts (i.e. negatively selected, autoimmune and non-autoimmune TCRs), we compared the distribution of experimentally-determined 1.D9.B2 hotspots with hotspots defined for the six other available TCR/MHC class II complex structures using *in silico* alanine scanning mutagenesis¹⁸⁻²¹ to generate an internally consistent dataset. This method calculates the approximate energetic contributions of all residues at a protein-protein interface by quantifying energy changes after computational replacement with alanine or glycine ($\Delta\Delta G$; hotspots are formally defined by $\Delta\Delta G$ values of greater than 1 kcal/mole).^{19,20} While this technique is not as accurate as directly determining $\Delta\Delta G$ from affinity differences of replacement mutations, it has been benchmarked and validated^{18,21} and is quite comparable to identifying hotspots by loss of cellular responses, as above for 1.D9.B2. Using these computational hotspot predictions, we mapped the distribution of calculated binding energy at each TCR/pMHC interface by superimposing the data on outlines of ligand/receptor footprints and representations of the molecular surfaces (Figure 2). The only consistent features identified in this analysis are the sets of diametrically opposed MHC anchor residues that may act as docking sites for the TCR. For the centrally-docked TCRs (D10, HA1.7 and 172.10), the majority of the binding energy contributed by the pMHC is focused on $\alpha 57$, $\alpha 61$ or $\alpha 62$ in the MHC α -chain and on $\beta 70$ in the MHC β -chain, residues that straddle the peptide binding groove near its middle and sit across the interface from the centrally clustered hotspots on the TCR. For the two autoimmune TCRs that are canted over the N-terminus of the MHC (3A6 and Ob.1A12), the major MHC hotspots similarly straddle opposing docking residues but at positions $\alpha 55$ or $\alpha 57$ and $\beta 81$ which are located in the N-terminal half of the binding groove. The P2 peptide residue, located toward the N-terminal end of the MHC binding cleft, appears to be a hotspot for two autoimmune TCRs (3A6: K5; Ob.1A12: H90), however, this position is also a hotspot for non-autoimmune D10 (R135). For the autoimmune 172.10 TCR, the P5 (Q3) residue positioned at the center of the binding groove is the only predicted hotspot. P3 residues are hotspots for the autoimmune 3A6 and Ob.1A12 TCRs (N6 and F91, respectively), in common with the non-autoimmune HA1.7 TCR (K310). TCR footprint boundaries (Figure 2A and 2B), while reflecting the N-terminal canting of 3A6 and Ob.1A12, do not differ between 172.10 and the non-autoimmune TCRs. Thus, although previous studies suggested that autoimmune TCRs are distinct in their focus on the N-terminus of the peptide functionally²² and structurally²³, our analyses suggest that there is no overall correlation between location of peptide hotspots and autoimmune versus non-autoimmune TCRs. Hotspot variations between non-autoimmune events are comparable to variations between autoimmune and non-autoimmune recognition. Comparing HA1.7 recognition of DR1 versus DR4^{24,25} shows that hotspots vary significantly with just MHC context: in the DR1 complex, P3 (K310) and P5 (N312) are predicted hotspots while P3 (K310) and P8 (K315) are hotspots, with different relative magnitudes, in the complex with DR4. From this detailed perspective, it is not possible

to cleanly distinguish between the autoimmune and non-autoimmune complexes based on hotspot magnitude or distribution.

Crystal structures of 1.D9.B2 reveal surprising CDR3 rigidity

To complement the biochemical data and distinguish, in the mutational analysis, between indirect and direct effects on pMHC binding, we determined the structure of 1.D9.B2 by x-ray crystallography (Table 1, Supplementary Figure 1A and Figure 3). The variable domains of the 1.D9.B2 TCR were cloned into a single chain format (scTCR). sc1.D9.B2 crystallized in space group $P2_1$ ($d_{\min} = 2.42 \text{ \AA}$) with four molecules (labeled AB, CD, EF and GH) in the asymmetric unit (AU). Initial experimental phases were determined by molecular replacement with another TCR structure.²⁶ The direct comparisons of independent views of sc1.D9.B2 revealed inherent substructure conformational flexibility (all four molecules were built independently and refined without using non-crystallographic symmetry (NCS) restraints in accordance with R_{free} calculations $\pm$ NCS). Such an analysis has not been possible before as all previous unliganded TCR crystal structures have only a single molecule in the AU. Two of the molecules in the AU, AB and CD, crystallized as a bivalent dimer or diabody²⁷ (Figure 3A), while molecules EF and GH crystallized as monobodies (Figure 3B). Although the linker is disordered for EF and GH, crystal packing considerations preclude these molecules from forming diabodies. Each domain in the TCR adopts an identical conformation (Supplementary Table 1) but the juxtaposition of these domains varies between the two formats (Figure 3C).

Electron density in the CDR3 β region (residues 97 through 102) was entirely absent in all four molecules in the AU, indicating considerable, uniform conformational flexibility here. These six residues (Leu97 β , Gly98 β , Gln99 β , Thr100 β , Asn101 β , Glu102 β) have not been modeled in any of the four molecules for this reason. However, the remaining five CDRs, including CDR3 α , display remarkable rigidity (Figure 3D), highlighted by comparison with the previously determined structure of another V α 2.3/V β 8.2 TCR (172.10; 87.4% identical to 1.D9.B2), specific for the complex of I-A^u with MBP Ac1-11 (pMHC Ac1-11).¹⁶ Superposition root mean square deviations (RMSD) on all atoms between the CDR1/2 loops of sc1.D9.B2 and 172.10 are all less than 1.6 $\text{\AA}$. Essentially, the only conformational variation in these five CDRs is the occasional use of alternate side-chain rotamers. Noting that the structure of TCR 172.10 was obtained when it was complexed with pMHC Ac1-11 , the extreme conservation of CDR conformation between sc1.D9.B2 and 172.10, and between multiple monobody/diabody views of sc1.D9.B2 in our unliganded crystal structure, strongly argues that five of the six CDRs in sc1.D9.B2 are quite constrained conformationally regardless of whether the TCR is bound to ligand. In contrast, the sixth CDR, CDR3 β , is more flexible in the 1.D9.B2 TCR than has previously been observed in any TCR crystal structure.

TCR CDR3 residues can affect pMHC recognition indirectly by rearranging TCR domains

In the scanning mutagenesis, most of the mutations that abolished recognition are distal from the predicted pMHC interface and are therefore not contributing directly to the interaction. For instance, the CDR1 α loop is stabilized by a triad of aromatic residues: Tyr24 α , Phe29 α and Phe32 α (Figure 3D). The Phe29 α Ser mutant failed to recognize either pMHC ligand, most likely by destabilizing the conformation of CDR1 α , affecting binding indirectly. Similarly, the mutated His29 β in the CDR1 β loop is directed towards the interior of the TCR, not towards pMHC. Along the CDR3 α loop, Lys99 α , Val100 α , and Ile101 α are also distant from the pMHC interface in a predicted complex, where only Tyr98 α is likely positioned for ligand contact. CDR3 β contains nine residues that ablate binding to both peptide/I-A^u complexes when mutated. Some of these residues (Arg103 β , Leu104 β , Phe105 β) are again far from the presumed recognition interface and are unlikely to be available for ligand contact. These distal residues are exposed to solvent but are involved in extensive main-chain β -strand pairing that maintains the anti-parallel structure of the loop base. In total, 12 of 18 mutations that abolished

recognition of pMHC ligands are predicted to do so indirectly and not through direct pMHC contacts.

Comparisons of the 172.10 and 1.D9.B2 structures revealed a feature of sc1.D9.B2 not seen in 172.10 or any other TCR structure: a prominent groove, up to 12 Å deep, that runs across the binding surface and one lateral face of sc1.D9.B2. This groove is the product of a significant twist in the packing of the V α domain against V β in sc1.D9.B2 relative to that of 172.10, structurally akin to cracking open a book. This twist is well beyond the limit of presumed 1.D9.B2 interdomain flexibility revealed by the comparison of the molecules in the sc1.D9.B2 AU, thus it cannot be accounted for simply by a molecule flexing more in one crystal than another. The change in V α /V β interdomain angle is not the result of a significant repacking of the aromatic cluster constituting the interface, which is structurally conserved in 172.10 and 1.D9.B2, but is instead due to sequence differences at gene segment junctions in CDR3 that result in loss of contacts at one edge of the V α /V β interface and gain of contacts at the opposite edge (Figure 3E). At position 93 α , serine (172.10) is replaced by isoleucine (1.D9.B2), with the loss of a hydrogen bond; at position 103 α , tyrosine (172.10) is replaced by lysine (1.D9.B2), no longer positioned to make a salt bridge with the conserved Asp59 β ; at position 104 α , glutamine (172.10) is replaced by valine (1.D9.B2), with the loss of another hydrogen bond; and His107 β (1.D9.B2) substitutes for Pro110 β (172.10), adding a hydrogen bond to the main-chain of Gly40 α (1.D9.B2) on the opposite side of the molecule. The importance of this observation is that sequence differences that alter V α /V β interdomain angle also have the potential to significantly affect ligand specificity by globally altering the overall arrangement of CDRs constituting the pMHC interface.

To start to investigate effects this V α /V β twist may have on ligand recognition, we generated pMHC125 for biophysical and crystallographic studies. Soluble pMHC125 was generated by fusing MBP125-135 in-frame to the N-terminus of the I-A^u β chain through a linker and adding C-terminal dimerization motifs.²⁸ The α - and β -chains of I-A^u were then co-transfected into S2 cells and secreted protein was purified as described.²⁸ sc1.D9.B2 binds to pMHC125 with an equilibrium dissociation constant of 11.6 μ M $\pm$ 0.3, determined by surface plasmon resonance measurements (Supplementary Figure 2), within the normal range of TCR/pMHC affinities. While attempts to co-crystallize sc1.D9.B2 and pMHC125 are ongoing, we determined the crystal structure of pMHC125 in isolation ($d_{\min}$ = 2.15 Å; Table 1, Supplementary Figure 1B and Figure 4). pMHC125 crystallized in space group P4₁2₁2 with one molecule per AU and was phased by molecular replacement using the pMHCAc1-11²⁸ structure as the search model. As expected, the overall structure of pMHC125 is very similar to that of pMHCAc1-11 (RMSD = 0.38 Å on 170 C α). Seven residues within the AB loop of the β 2 domain are disordered and were not modeled. Both MBP peptide backbones adopt nearly identical type II polyproline conformations (RMSD = 0.29 Å on 12 C α ; Figure 4B). Promiscuous peptide binding among I-A molecules has been attributed to partially empty MHC pockets.²⁹ Here we find that only one of four pockets in the groove is significantly occupied by MBP125-135 peptide side chains (Tyr6 in the P6 pocket). The P1, P4, and P9 pockets contain well-ordered water molecules and contribute to stability, as has been seen in the pMHCAc1-11 structure.²⁸

Previous mutagenic data using a panel of MBP125-135 analog peptides and several MBP125-135/MBP136-146 bispecific hybridomas identified the centrally located aspartic acid at P5 as essential for ligand recognition.⁴ In the pMHC125 structure, the Asp-P5 side-chain is engaged in a salt bridge with a lysine at peptide position 7, partially occluding these side-chains from hypothetical interactions with a TCR. We predict that this salt bridge is disrupted in order for TCRs to fully engage Asp-P5. Such adjustments in pMHC ligands upon binding have precedents; for example, Lys-P2 in the MBP/HLA-DR2a complex significantly alters its conformation upon binding to TCR 3A6.²²

Different pMHC docking modes are utilized by sc1.D9.B2 and 172.10

Because 1.D9.B2 and 172.10 TCRs are highly conserved in terms of sequence, variable gene segment utilization and CDR structure, we wished to compare the interactions of 1.D9.B2 and 172.10 with their peptide/I-A^u ligands. The 1.D9.B2/pMHC125 ternary complex was modeled by two different methods. sc1.D9.B2 and pMHC125 were initially docked onto each other using the 172.10/pMHCAc1-11 complex structure as a scaffold. However, we find that the sc1.D9.B2 α/β interdomain angle observed for both the monobody and diabody forms results in severe steric clashes between the TCR V α domain and pMHC in a complex modeled by simple docking. Therefore, given the apparent rigidity of 1.D9.B2 CDRs and limited interdomain flexibility, we conclude that 1.D9.B2 interacts with pMHC125 in a manner distinct from that observed in the 172.10/pMHCAc1-11 complex structure. This finding confirms the significant impact of indirect effects of distal junctional sequence differences on global binding site arrangement through altered interdomain angles.

Because 1.D9.B2 cannot dock onto I-A^u in the 172.10 mode, the sc1.D9.B2/pMHC125 complex was then modeled by the more sophisticated computational methods available in RosettaDock.³⁰ The most prominent feature of this alternately-modeled complex is an overall rotational shift of sc1.D9.B2 relative to pMHC125 in order to eliminate steric clashes (Figure 5A). The crossing angle³¹ for sc1.D9.B2 is 57°, far more orthogonal than has been seen for other TCRs except Ob.1A12. In this modeled sc1.D9.B2/pMHC125 complex, 2360 Å² of solvent-accessible surface area is buried, with the TCR V α domain contributing 54% of the interface, comparable to the 53% contributed by the TCR V α domain in 172.10/I-A^u/MBPAc1-11. Ligand recognition is mediated by nine TCR residues (Figure 5B). Of these nine, five were mutated by our experimental mutagenesis and were all shown to be critical for cellular response (α Y98, α K99, β N31, β G98, β Q99; Figure 1), confirming some aspects of the modeled complex. The modeled CDR3 β loop overlies the peptide from the P5 to P8 positions, consistent with the experimental observation that mutation of any solvent-exposed CDR3 β residue ablates response, with the major TCR contacts focused on Arg-P2 (from CDR3 α) and Ser-P8 (from CDR3 β). The presence of an arginine at the P2 position in pMBP125 and a glycine in the same position in pMBPAc1-11 (labeled P-1 in that structure) independently dictates that 1.D9.B2 cannot engage pMHC in the same docking mode as 172.10, because the arginine would sterically clash with the rigid CDR1 β if 1.D9.B2 used the 172.10 docking mode. These constraints may contribute to the rotation of overall docking angle predicted for sc1.D9.B2. This model also strongly suggests that the V α 2.3/V β 8.2-expressing 1.D9.B2 and 172.10 TCRs interact very differently with pI-A^u complexes, in part due to differences in the interface between the α - and β -chain V domains. However, one caveat to this model is that our biological data showed that substitution of Asp-P5 with alanine abolished T cell responses but the model indicates a lack of direct hydrogen bonds or other significant interactions between Asp-P5 and sc1.D9.B2 in the model (the Asp-P5/Lys-P7 salt bridge is retained in the modeled complex, though it was not constrained to do so).

TCR interdomain interfaces can twist to accommodate multiple pMHCs

The apparent constraints imposed by the sc1.D9.B2 interdomain angle on pMHC docking led us to examine how the V α /V β interdomain angle varies in other TCR/pMHC complexes. Using a rigorous mathematical description of the rotational relationship between TCR V domains, we analyzed sc1.D9.B2 and the 35 TCR structures currently available (Supplementary Table 1 and Figure 6). Here, the interdomain rotational relationship is broken down into three components: two angles defining the pitch of the pseudodyad axis relating the two domains (ω , ϕ) and a third angle defining the rotation around this axis, around 180°, defining the best superposition of V β onto V α (χ). These analyses reveal a wide range of values for the different TCR structures, with pseudodyad pitches varying by more than 20° and χ values ranging from 165° to 190°. Thus, different TCRs can clearly adopt different interdomain orientations,

comparable to the behavior of antibodies.³² Interestingly, the angle differences between sc1.D9.B2 and 172.10 appear to be due in large part to only a few specific sequence differences, which significantly affect TCR/pMHC docking. Interdomain angles for individual TCRs also clearly flex from unbound to bound states (D10, 1G4, KB5C20 or ELS4) and between complexes of one TCR with different pMHCs (BM3.3). Both axis pitch and χ can change between unbound TCR and bound to different ligands. There is, as yet, no clear correlation between the magnitude or direction of these changes and measured TCR/pMHC thermodynamic parameters, but the availability of such data is limited. However, these results show that TCR interdomain angle variation between different TCRs, influenced by CDR junctional variation, and the range of interdomain flexibility available to any given TCR are previously unrecognized, but potentially significant, aspects of TCR specificity and polyspecificity respectively.

Discussion

TCR/pMHC interactions have long been recognized as displaying some degree of polyspecificity. Hypotheses regarding mechanisms enabling such degeneracy often focus on single parameters, such as whether degeneracy occurs when interactions focus primarily on either the peptide alone or the MHC alone, or whether different pMHC ligands are accommodated primarily by CDR conformational plasticity or by grossly different docking orientations of the TCR on the pMHC. A recent report of experimental binding energy maps for TCR/pMHC interfaces concluded that TCR/pMHC binding is a function not only of positive interactions but of a lack of negative interactions (steric clashes) at the TCR/pMHC interface.^{10,33} Here we demonstrate that TCR residues distant from the interface can also affect TCR recognition using a novel mechanism, i.e., inducing V region interdomain rearrangements that mediate global effects on the TCR interface. While four of six CDR loops are conserved in structure and sequence between the two functionally distinct 1.D9.B2 and 172.10 TCRs, the CDR3 sequence differences impart specificity in two ways. In addition to contributing distinct pMHC contact residues, differences in CDR3 non-contact residues indirectly remodel the pMHC recognition surface by affecting the global arrangement of α -chain CDRs relative to those in the β -chain. While it has been previously recognized that non-contact TCR residues can stabilize specific CDR loop conformations or enable varying degrees of CDR flexibility in antibodies, the major role demonstrated here for CDR3 residues in affecting α/β chain pairing is consistent with the fact that these hypervariable residues comprise over 30% of the α/β domain interface. The residues that appear most critical in defining the different interdomain angles for 1.D9.B2 and 172.10 are CDR3 junctional sequences. The ability of these distal, non-germline-encoded residues to alter the $V\alpha/V\beta$ interface angle thus reveals another mechanism for dramatically enlarging potential TCR recognition space.

Our analyses of $V\alpha/V\beta$ pairing angles for the available TCR MHC class II-restricted structures suggest a previously unrecognized mechanism for polyspecificity. Interdomain angles were found to vary not only between different TCRs but also for individual TCRs bound to different pMHC ligands (e.g. BM3 structures). Thus, the ability to adopt different $V\alpha/V\beta$ pairing angles directly allows TCRs to generate different surfaces available for pMHC interaction, recapitulating the indirect effect of junctional diversity on antibody antigen combining sites.

Another recent report of experimental binding energy maps for TCR/pMHC interfaces (Felix et al., 2007) concluded that a particular family of polyspecific, allogeneic TCRs appear to use conserved docking modes on pMHC ligands, echoing the apparent dominance of a particular docking mode for $V\beta 8.2$ TCRs on MHC class II proteins based on purely structural studies. The unusually constrained conformations of CDR1 and CDR2 in sc1.D9.B2 argue for these elements contributing rigidly to the docking of this TCR to its ligands, which is consistent with the two-step model of TCR binding.³⁴ However, the predicted docking mode for sc1.D9.B2,

which expresses V β 8.2, is not obligate as 172.10 exhibits strong sequence similarity in CDRs 1 and 2 but docks differently onto I-A^u. Thus, while obligate use of restricted docking modes would inherently limit the polyspecificity of TCRs, our data indicate that this restriction is relieved by CDR sequence variation at positions significantly distant from the pMHC interface, emphasizing the need to view TCR recognition specificity as a global property of the molecule.

While additional crystal structures are needed to fully parse out 1.D9.B2 interactions, these data introduce a novel perspective on TCR degeneracy. The ability of variable CDR residues distant from the actual pMHC binding surface to dramatically alter ligand binding echoes comparable effects long understood to be in play in antibody-mediated recognition events. Antibodies are viewed as having sometimes plastic ligand-binding surfaces, composed of CDR loops with varying degrees of flexibility, arranged in an array that is organized by more distant residues that mediate effects on individual loop structure or overall assembly of loops on the surface. Our studies suggest this description also applies to TCRs. Utilization of such parallel mechanisms suggests that the evolutionary selection of an antibody-like fold for TCRs may not so much reflect an inherent preference of germline-encoded TCRs for pMHC ligands, but rather an antibody-like ability to generate a huge diversity of binding specificities, which can then be filtered during development to select those that meet particular physiological requirements.

Materials and Methods

Experimental mutational scanning mutagenesis

cDNAs encoding the α - and β -chains of 1.D9.B2 were cloned into the pMI retroviral vector³⁵; single amino acid changes were introduced using the QuikChange Site-Directed Mutagenesis kit (Stratagene) following the manufacturer's protocols. Residues were replaced with alanine (D, E, F, H, K, N, Q, R, T, Y) or serine (A, G, I, L, V). Mutated and wild-type TCR α - and β -chain encoding plasmids were transfected into the ϕ NX-Ampho retroviral packaging cell line (the gift of P. Achacoso and G. Nolan) using Ca₃(PO₄)₂ precipitation as previously described.³⁶ Retrovirus-containing supernatants were used to transduce TCR⁻ DO11.10 hybridoma cells³⁷ by resuspending 5×10^5 log-phase cells in 1 mL supernatant plus 5 μ g/mL polybrene. Clones stably expressing the parent α - and β -chains were transduced with retrovirus encoding cognate wild-type or mutant α - or β -chains. After ~ 1 week, V α 2.3⁺, V β 8.2⁺ and CD4⁺ transduced cells were sorted and subcloned. Bulk cultures of each hybridoma and expanded subclones with comparable levels of V α 2.3, V β 8.2 and CD4 were tested for IL-2 production after stimulation with 1.9 and 0.95 μ g/mL whole MBP, 6 and 2 nM MBP121-140 and 50 μ M MBP131-150 as described previously.¹⁵ Student's *t*-test was used for determining *P* values with *n*=4 for both mutations analyzed (Y24A and N26A).

Protein production

A soluble scTCR construct was engineered encoding the variable domains of the 1.D9.B2 hybridoma (α -chain residues 20 through 132 and β -chain residues 30 through 139) through a 15 residue long linker (GSADDAKKDAKKDG), identical to the linker used for generating another scTCR²⁶, connecting the C-terminus of the β -chain to the N-terminus of the α -chain. The scTCR construct was subcloned into the NdeI and BamHI restriction sites of the pET22b expression vector (Novagen) and transfected into the BL21 Codon+ *E. coli* strain (Stratagene). Protein was expressed as inclusion bodies which were solubilized in 8 M urea, 100 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 4 mM reduced glutathione, 0.4 mM oxidized glutathione, 0.5 mM PMSF and 50 mM glycine. Protein, at 0.5 to 1 mg/mL, was refolded by dialysis versus 50 mM Tris-HCl pH 8.0, 2 mM EDTA, 400 mM L-arginine and urea, which was decreased in concentration two-fold every 24 hours to 0.5 M urea, when the dialysant was switched to 150 mM NaCl, 1 mM EDTA, 0.02% NaN₃, and 25 mM PIPES pH 7.4 (PNEA). Soluble protein at

this stage was concentrated to 2 to 4 mg/mL before final purification by size exclusion chromatography (SEC) on a Superdex 75 column (Amersham Biosciences). Yields ranged from 0.5% to 2% from inclusion bodies to final, concentrated protein. pMHC125 was produced analogously to pMHCAc1-11¹⁶, with a linker (GSGSGS) introduced to join the C-terminus of the MBP peptide to the N-terminus of the β -chain of I-A^u (I-A^u expression vectors were the generous gift of Chris Garcia). Proteins were validated by reducing/non-reducing SDS-PAGE analysis to confirm proper disulfide bond formation and analytical SEC to confirm solution monodispersity.

Surface plasmon resonance affinity analysis

pMHC125 was coupled to a research-grade CM5 chip (Biacore) to a final coupled RU of 2394 using standard amine coupling chemistry following the manufacturer's protocols. sc1.D9.B2, repurified by SEC less than 24 hours prior analysis, was used as the analyte at six separate concentrations with randomly interspersed blank buffer runs at a flow rate of 30 μ L/min. All measurements were conducted at 298 K. Raw sensorgrams were corrected using Myszka double-subtraction³⁸ against the blank channel response and eight averaged blank buffer injections. Data from 160-180 seconds after injection were selected for analysis. Averaged data were fit to the equation $\text{Response} = ((K_a)([1.D9.B2])(R_{\text{max}}))/(K_a([1.D9.B2] + 1)$ using the Prism software package.

Crystallography

sc1.D9.B2 TCR crystals grew over 4 to 5 days by vapor diffusion from drops containing 1 μ L of protein (at 3 mg/mL) in PNEA plus 1 μ L of well solution (12% w/v PEG 6000, 100 mM HEPES pH = 7.5 and 3% v/v MPD) at 4°C. sc1.D9.B2 crystals were cryocooled at 100 K in well solution plus 25% glycerol. Diffraction data were collected at beamline 5.0.1 (Advanced Light Source) and were processed using DENZO-SCALEPACK³⁹ in space group P2₁ (Table 1). The structure was phased by molecular replacement using TCR scD10 (PDB ID: 1D9K) as a search model with the program AMoRe as implemented in the CCP4 suite.³⁹ All eight chains of sc1.D9.B2 were built independently into Fo-Fc difference electron density maps using the programs Xfit⁴⁰ and Coot⁴¹ and refined using Refmac5 (as implemented in CCP4; final Ramachandran statistics: 90.1% most favored, 9.9% additional allowed). NCS restraints were applied to the α - and β -chains independently excluding all six CDR loops in all four molecules in the AU.

pMHC125 crystals grew over the course of 1 week by vapor diffusion from drops containing 1 μ L of protein (at 12.6 mg/mL) in PNEA plus 1 μ L of well solution (0.1 M NaOAc pH 4.6, 25% w/v PEG 1000) at 18 °C. pMHC125 crystals were cryocooled at 100 K in well solution plus 25% glycerol. Diffraction data were collected and processed as above but in space group P4₁2₁2 (Table 1). The structure was phased using molecular replacement with AMoRe, using the pMHCAc1-11 structure (PDB ID: 1U3H) as a search model, and built and refined as above (final Ramachandran statistics: 88.6% most favored, 10.8% additional allowed, 0.6% generously allowed). TCR V α /V β interdomain relationships were determined by superimposing the V β domain onto the V α domain using THESEUS⁴² and then calculating the rotations relating the two orientations in spherical polar coordinates. (ω , ϕ , χ).

Computational docking

The structures of sc1.D9.B2 and pMHC125 were computationally docked with RosettaDock.³⁰ The residues comprising the CDR3 β loop and an additional flanking residue from each side (in total, residues Asp96 β -Arg103 β) were allowed to sample backbone conformations using the fragment-insertion approach of Rosetta.⁴³ The initial relative configuration of pMHC and TCR α and β was determined by superimposing these partners on the corresponding molecules in the 172.10/pMHCAc1-11 complex structure (PDB ID: 1U3H). Conformation space was

sampled around this initial conformation using a Monte-Carlo procedure with random rigid-body perturbations of at most 3 Å of the distance between the monomers' centers of mass, 8 Å in the plane perpendicular to the axis connecting these centers and 10 degree rotations of the two proteins as rigid bodies. Decoys were ranked according to the Rosetta full-atom energy, which is dominated by Lennard-Jones interactions, an implicit solvation model and an orientation-dependent hydrogen-bonding potential.³⁰ 7000 decoys were thus generated, scored and the lowest-energy 200 decoys were clustered according to RMSD. The lowest-energy representatives of each of the 10 lowest-energy clusters were visually examined according to the physical interactions formed across the interface and the extent to which the interactions resembled other TCR pMHC interactions. The final cluster was selected from this panel based on stereochemistry and agreement with experimentally determined biochemical data.⁴ To provide some assessment of the capability of the procedure described above to identify near-native conformations for similar TCR/pMHC interactions, we used the same docking procedure on the complex in 1U3H, removing the CDR3β loop, and allowing it to sample backbone conformation space using fragment insertion. The top-scoring cluster in this analysis was very close to the native state structure with RMSD of 1.05 Å.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Scanning mutagenesis analysis of 1.D9.B2 interacting with pMHC125 and pMHC136
 (A) Parental and mutant 1.D9.B2 hybridomas were tested against APCs pulsed with whole MBP, MBP121-140 or MBP131-150 as indicated. Responses are shown as a ratio of mutant to wild-type (parent) responses. Mutations that reduced responses to less than 15% of the parental response (threshold indicated by the dotted line) are considered to have abrogated the response and are therefore defined as hotspots. P-scores for two selected pairs of responses are also shown. (B) The sequence alignment of the variable domains of 172.10 and 1.D9.B2 is shown, with CDR regions indicated and sequence differences boxed. Sequence differences outside CDR3 regions are mutations engineered to increase sc172.10 solubility.

Figure 2. Analysis of TCR/pMHC class II interfaces

(A) $\Delta\Delta G$ and footprint analyses of the interfaces of all six TCR/MHC II complexes currently available are mapped onto molecular surfaces of the TCR and pMHC from each complex, colored by domain (TCR α : light yellow; TCR β : light orange; MHC α : dark blue; MHC β : light blue; and peptide: green). Each complex has been splayed open to reveal interface features, with each partner in a complex viewed from the perspective of the cognate partner. For each complex pair, the TCR is shown above the MHC. Hotspot symbols (circles for TCR residues, squares for pMHC residues) are scaled to the magnitude of the contribution (ranging from 1 kcal/mol to 7 kcal/mol), labeled by residue ID and colored according to chain identity. For each surface, the footprint of the binding partner is also outlined as an irregular blob, colored

by the molecule contributing the contact: TCR α in yellow, TCR β in orange, MHC α in dark blue, MHC β in light blue and the peptide in green. (B) Overlay, based on MHC α -chain superpositions, of the minimum bounding rectangles for TCR footprints on pMHCs. Bounding boxes of TCR footprints from non-autoimmune contexts are shown in orange and autoimmune contexts in green. The center of each box is indicated by a cross. (C) 1.D9.B2 experimental hotspots (Figure 1) are mapped onto the sc1.D9.B2 structure for comparison to the data above (molecular surface colored as in (A); residues where mutations abolish recognition of both ligands are shown as hotspot symbols colored as in (A)). The structure of the disordered CDR3 β loop and the footprint of pMHC125 are based on the computationally-docked model (Figure 6). Residues that are distal from the recognition surface are labeled in italics and residues on the recognition surface are labeled in boldface.

Figure 3. Structure of sc1.D9.B2

(A) Ribbon representations of the structures of molecules AB and CD (shown in shades of green or blue, respectively), which together form the diabody present in the AU, are shown; secondary structure elements are shown as arrows (β -strands) and coils (α -helices). Ordered sections of the introduced linker peptides, corresponding to thirteen of fifteen residues total for AD and all fifteen residues for CB, are shown in red; disordered sections of the structure are shown as dashed red lines. (B) Ribbon representations of the structures of molecules EF and GH, which crystallized as monobodies, are shown in shades of orange and red, respectively. Positions of CDR loops are additionally highlighted with CDR1 loops are in blue, CDR2 loops in grey, and CDR3 loops in green. The two molecules have been superimposed to emphasize

structural conservation. (C) Superposition of all four molecules in the asymmetric unit highlights a small difference in the α/β interdomain angle between the diabody (in shades of green) and monobody (shades of orange) forms of TCR 1.D9.B2 in the AU, with the diabody molecules displaying a slightly more extreme α/β interface angle. (D) The conservation of CDR structure is shown by all-atom representations of superpositions of the four views of each 1.D9.B2 CDR along with the corresponding element from 172.10 where applicable. 1.D9.B2 CDRs are colored to indicate results of the scanning mutagenesis (wild-type level response by mutant: blue; ablation of response: red; and not tested: black); 172.10 CDRs are colored by atom type (carbon: light gray; nitrogen: blue; oxygen: red). For CDR1 α , CDR2 α , and CDR1 β , TCR 172.10 (shown in white) adopted the same conformation. (E) Four CDR3 amino acid changes between 1.D9.B2 and 172.10 result in the loss or gain of interdomain contacts. Residues labeled in red (α 93, α 103, α 104) represent loss of contacts along the groove face whereas the last panel, labeled in green, highlights a gain of contact (β 107/110) on the face opposite the groove. In the panels, 1.D9.B2 is depicted in yellow (α chain) and orange (β chain). Light green (α chain) and dark green (β chain) residues are from 172.10.

Figure 4. Structure of pMHC125 alone and docked onto sc1.D9.B2

(A) Ribbon representations are shown of the structure of pMHC125 (viewed from the perspective of a TCR). Peptide residues that are available for TCR contact are shown in red, peptide residues occupying shallow surface pockets are shown in green, and peptide residues that serve as MHC anchors are shown in blue. (B) Structural comparison of MBPAc1-11 (gray) and MBP125-135 (green) when bound to I-A^u. (C) Computationally docked model of 1.D9.B2/pMHC125 complex using RosettaDock. MHC α and β chains are depicted in dark blue and light blue respectively with peptide residues in green. TCR chains are shown in yellow (α) and orange (β).

Figure 5. Computational docking of sc1.D9.B2 onto pMHC125

(A) CDR loops for both 172.10 and 1.D9.B2 are shown on I-A^u (CDR1: blue; CDR2: red; CDR3: green; 1.D9.B2 in darker shades and 172.10 in lighter shades). Despite highly conserved loop structures, rotation of the α chain and opening of a groove precludes similar docking on the conserved MHC residues. Computational modeling of 1.D9.B2 with pMHC125 predicts a $\sim 14^\circ$ rotation in order to accommodate both structural ($\alpha\beta$ interface) and peptide differences (MBP125-135 v. MBPac1-11). (B) A contact map derived from the modeled 1.D9.B2:pMHC125 complex highlights TCR residues (shown in orange and yellow circles) making specific interactions to pMHC.

Figure 6. TCR Va/Vβ interdomain angle variation

(A) Ca backbone representations of the structures of scD10 ($\text{Va}2/\text{V}\beta 8.2$; specific for I-A^k/CA), sc172.10 ($\text{Va}2.3/\text{V}\beta 8.2$) and all four independent views of sc1.D9.B2 ($\text{Va}2.3/\text{V}\beta 8.2$) are shown superimposed on their $\text{V}\beta$ domains (in shades of gray; Va domains are colored as indicated) to highlight the effect of α/β interdomain angle and rotational axis variation. (B) TCR variable domain cassette pseudodyad axes are plotted to graphically highlight distinctions, labeled by TCR name and complex state (U: unbound or B: bound); individual TCRs are grouped by color, with abbreviated labels in a single, matching color if more than one structure is available. For cases where multiple ligand complex structures are available, TCRs are labeled as X1, X2, etc., as listed in Supplemental Table 1. Variation in the pitch of

the pseudodyad axes (ω , ϕ ; Supplemental Table 1) is shown by plotting the intersection of each TCR's associated axis with an arbitrary plane, roughly parallel to the pMHC binding surface (the location of the axes emanate from a single point at the center of mass of the D10 TCR). As a reference, a schematic outline of an idealized TCR is shown ($V\alpha$ domain on the left (yellow) and $V\beta$ domain on the right (orange)). (C) Variation in the rotation around pseudodyad axes (χ) is plotted, with TCRs labeled as in (B); χ values have been multiplied by five to highlight distinctions. The variation in χ between sc1.D9.B2 (red), 172.10 (orange), and D10 (yellow) is highlighted by coloring the labels; extreme variation in other TCRs is highlighted by boxing labels and coloring lines (1G4 in cyan, ELS4 in dark blue, KB5C20 in green and BM3.3 in light blue).

Table 1

Data collection and refinement statistics

	1.D9.B2	pMHC125
Data collection		
Space group	P2 ₁	P4 ₁ 2 ₁ 2
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	71.6, 105, 81.5	104, 104, 97.2
α , β , γ (°)	90, 116, 90	90, 90, 90
Wavelength (Å)	1.00	1.00
Resolution (Å)	40.4-2.42 (2.51-2.42) *	46.4-2.15 (2.23-2.15) *
<i>R</i> _{sym} or <i>R</i> _{merge}	4.1 (19.6)	8.9 (28.9)
<i>I</i> / σ <i>I</i>	24.0 (6.29)	18.7 (5.99)
Completeness (%)	99.1 (96.4)	99.9 (99.7)
Redundancy	4.1 (3.2)	13.8 (13.7)
Refinement		
Resolution (Å)	2.42	2.15
No. reflections	39252	29462
<i>R</i> _{work} / <i>R</i> _{free}	0.182/0.253	0.205/0.265
No. atoms	7242	3203
Protein	6915	2997
Ligand/ion	0	0
Water	327	206
<i>B</i> -factors		
Protein (Å ²)	29.2	27.9
Water (Å ²)	31.3	28.9
R.m.s deviations		
Bond lengths (Å)	0.023	0.023
Bond angles (°)	2.53	1.90

Data were collected from single crystals for each structure

* Highest resolution shell is shown in parentheses