Structural interplay between germline interactions and adaptive recognition determines the bandwidth of TCR-peptide-MHC cross-reactivity

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The T cell antigen receptor (TCR)–peptide–major histocompatibility complex (MHC) interface is composed of conserved and diverse regions, yet the relative contribution of each in shaping recognition by T cells remains unclear. Here we isolated cross-reactive peptides with limited homology, which allowed us to compare the structural properties of nine peptides for a single TCR–MHC pair. The TCR’s cross-reactivity was rooted in highly similar recognition of an apical ‘hot-spot’ position in the peptide with tolerance of sequence variation at ancillary positions. Furthermore, we found a striking structural convergence onto a germline-mediated interaction between the TCR CDR1α region and the MHC α2 helix in twelve TCR-peptide-MHC complexes. Our studies suggest that TCR-MHC germline-mediated constraints, together with a focus on a small peptide hot spot, might place limits on peptide antigen cross-reactivity.
TCR cross-reactivity has also been studied extensively, with divergent conclusions. On the one hand, TCRs have the ability to 'see' many different peptide antigens presented by MHC12,30–32. On the other hand, upon close inspection, TCRs may be less cross-reactive than previously appreciated, as most cases of cross-reactivity seem to be explained by preservation of several key TCR contact residues in seemingly non-homologous peptides23,30,33.

Published work has considered TCR germline bias for MHC, cross-reactivity, and signaling independently. Taking into account structural and energetic inter-relationships might prove beneficial for full understanding of TCR-pMHC recognition and signaling. Here we used the mouse 42F3 TCR, which recognizes the MHC class I molecule H-2Ld (ref. 1), as a model system to further clarify the interplay between TCR cross-reactivity and germline specificity. We used pMHC libraries displayed on yeast to screen recombinant, multimeric 42F3 TCR in a cell-free environment, free of any constraints on binding, and isolated recognized peptides with limited homology to the cognate antigen.

By characterizing the binding, signaling and structural properties of TCR-pMHC complexes with limited homology, we found that despite diversity in the peptide sequences it recognized, the 42F3 TCR maintained very similar 'hot-spot' contacts with the most prominent up-facing peptide residues, which resulted in a high degree of cross-reactivity with retention of specificity for key positions. Furthermore, while the TCR adjusted its binding mode to engage different peptides, rather than 'seeing' a spectrum of disparate binding solutions, the Vβ domain of the TCR ratcheted between two 'preferred' positions over the MHC α1 helix while retaining interaction between the MHC α2 helix and the TCR Vα that was almost completely superimposable in 12 structures of Vα3 TCR with H-2Ld. On the basis of these studies, we propose a model that functionally integrates conserved interactions between TCR germline regions and MHC helices and peptide cross-reactivity in which genetically imprintied biases toward the MHC help to 'steer' TCR binding solutions but still permit the structural adaptability needed for cross-reactivity.

**RESULTS**

A second-generation, yeast-displayed H-2Ld

Peptide libraries displayed by the mouse MHC class I molecule H-2Ld on yeast have been developed that are able to identify pMHC ligands for the alloreactive H-2Ld-specific TCR 42F3 (ref. 1). These libraries, based on the 'mini-MHC' α1x2 scaffold H-2L4-m31 (ref. 34), use a single-chain configuration in which the carboxyl terminus of the mini-MHC is fused to the amino terminus of the nine-amino acid peptide1. Selection identified epitopes that mimic ('mimotopes') the cognate antigen QL9 (amino acid sequence, QLSPFPFDL), but all agonists identified shared obvious sequence homologies with QL9 and, upon crystallographic study, shared very similar TCR docking footprints3. We suspected the scaffold design might have limited the recovery of diverse peptide sequences, indicated by the very weak staining of H-2Ld-m31 fused to QL9 (QL9-m31) by 42F3 TCR tetramers1.

Since we sought to find peptide sequences with greater divergence from the cognate antigen QL9 to better 'stress test' TCR-pMHC recognition in the face of alternative peptide-recognition chemistries, we developed a second-generation 'mini-MHC' yeast display scaffold linking the carboxyl terminus of the peptide to the MHC molecule (Fig. 1). The MHC molecule included substitution of alanine for the tyrosine at position 84 that opened a path at the carboxy-terminal end of the groove to accommodate a linker (Supplementary Fig. 1), as originally shown for pMHC single-chain trimers35. However, in this orientation, the carboxyl terminus of the peptide was far from the amino terminus of the MHC molecule and would require a long connecting linker. Therefore, we circularly permutated the MHC molecule to relocate the amino and carboxyl termini of the MHC molecule closer to the carboxyl terminus of the peptide (Supplementary Fig. 1).

To circularly permute the MHC, we interrupted the sequence at a loop near the carboxyl terminus of the peptide so that H-2Ld residue 120 became the new carboxyl terminus and Cys121 became the new amino terminus, while at the same time we fused the former carboxy-terminal residue 180 to the former amino-terminal residue 1 with a short glycine-serine linker (Supplementary Fig. 1). To avoid spontaneous formation of intermolecular disulfide bonds, we altered the new amino terminus with substitution of serine for the cysteine at position 121. The peptide was then appended to the new amino terminus to complete the circular permutation (QL9-m31 r-CP). While yeast expressing QL9-m31 r-CP stained positively for

![Figure 1](image-url)
a c-Myc epitope tag, they were not recognized by either 2C TCR tetramers or 42F3 TCR tetramers (Supplementary Fig. 1b), which suggested that QL9-m31r-CP was displayed on the yeast surface but was incorrectly folded. To restore the native fold, we evolved the m31r-CP design by creating a pool of ~1 × 10^8 variants produced by error-evolved the m31r-CP design by creating a rectly folded. To restore the native fold, we displayed on the yeast surface but was incor - which suggested that QL9-m31r-CP was

Supplementary Fig. 1b

clones from a library of 4.2 × 10^8 peptide variants (replacement of tyrosine for the aspartic acid at position 122 and alanine at position 136) in this scaffold variant occurred in the proximity of the linker-MHC junction, which perhaps accommodated the artificial linker or stabilized the new MHC fold-initiating sequence (Supplementary Fig. 1c).

We found a subset of circularly permuted scaffold clones that contained only interface and peptide-distal substitutions and recognized both 2C TCR tetramers and 42F3 TCR tetramers. The clone with the brightest TCR tetramer staining was m31r-CP-E3 (Supplementary Fig. 1c). The two substitutions selected (replacement of tyrosine for the aspartic acid at position 122 and replacement of threonine for the alanine at position 136) in this scaffold variant occurred in the proximity of the linker-MHC junction, which perhaps accommodated the artificial linker or stabilized the new MHC fold-initiating sequence (Supplementary Fig. 1c). We proceeded to construct new libraries to select for peptides presented by the circularly permuted MHC scaffold.

Peptide specificity of the 42F3 TCR

We created a 'random' peptide library tethered to m31r-CP-E3 (Fig. 1a and Supplementary Fig. 1a–d). The diversity of this 'random' nine-amino acid library was limited at anchor positions P2 (proline) and P9 (phenylalanine, isoleucine, leucine and methionine) to reflect the natural 'preference' of H-2Lα-presented peptides36 (Fig. 1b). For selection, we used streptavidin-coated magnetic beads30 saturated with biotinylated 42F3 TCR to allow enrichment of yeast clones from a library of 4.2 × 10^8 peptide variants (Fig. 1b,c and Supplementary Fig. 1c). We recovered a range of peptides different in sequence from each other and from the native agonist QL9 (Fig. 1c,d and Supplementary Fig. 1f). We sequenced several hundred clones from the final selected pool and observed a high degree of sequence diversity in all positions except positions P6 and P7. P7 was uniformly a large hydrophobic residue, as in QL9 (Fig. 1c,d). P6 exhibited a strong bias toward proline and glycine, as in QL9, but allowed substitution with tryptophan and glutamic acid (Fig. 1c,d). The P5–P6–P7 sequence stretch in QL9 forms an arch that peaks at P7 and most intimately contacts CDR3β through apolar and van der Waals interactions3. Collectively, there seemed to be selective pressure to preserve this interaction mode while allowing diverse chemistries at other positions (Fig. 1c,d). We sub-classified the sequences into related families and synthesized ten peptides for characterization, including five for structural characterization, whose sequences were suitably divergent from each other, from the cognate antigen QL9, and from the peptides derived from the published libraries1.

Signaling properties of library-selected peptides

We screened CD8^+ and CD8^- 42F3 T cells for their production of interleukin 2 (IL-2) after stimulation with antigen-presenting cells presenting a subset of divergent peptides that arose from the second-generation selections and compared them to the QL9 mimotopes that arose from the first-generation libraries (Fig. 2a). We transduced 58α^-β^- mouse T cell hybridoma cells to express the 42F3 TCR and assessed their IL-2 responses to peptides presented at a dose of 10 μM on antigen-presenting cells. Unlike the lone non-agonist peptide selected (p3A1; sequence, SPLDSDLWW1) from the first-generation library, the majority of peptides selected from the second-generation library elicited substantial IL-2 responses in these cells (Fig. 2a). The synthetic peptide antigens had a range of dependence on the co-receptor CD8 on the basis of our initial screen, as several second-generation peptides failed to stimulate or weakly stimulated cells in the absence of CD8 at this high peptide dose (Fig. 2a).

We titrated the IL-2 responses of CD8^- 42F3 T cells for various agonist peptides with diverse sequences. For this set, we observed responses to IL-2 across a several-log range of EC50 values (effector concentration for a half-maximum response) (Fig. 2b, Table 1 and Supplementary Fig. 2). Additionally, we assessed the affinity and
kinetics of the binding of the 42F3 TCR to each ‘titrated’ peptide by surface plasmon resonance (Fig. 2b, Table 1 and Supplementary Fig. 3a–c). As expected, we found that the 42F3 TCR–pMHC complexes with the highest affinity in the second-generation libraries were potent stimulators of IL-2 responses, but we also observed high-affinity ligands (pCPC5 and pCPA12) that produced partial responses. We were potent stimulators of IL-2 responses, but we also observed kinetics of the binding of the 42F3 TCR to each ‘titrated’ peptide by surface plasmon resonance (Fig. 2b). We were able to sub-divide the 42F3 TCR agonists into two categories. We defined ‘potent agonists’ as those peptides that elicited equally strong IL-2 responses in the presence of CD8 and absence of CD8 in our screen, and ‘CD8-dependent agonists’ as those that required CD8 to maximally stimulate IL-2 production (Fig. 2a). While the potent agonists all produced high maximal IL-2 responses, the CD8-dependent agonists elicited a range of maximal responses, including high (p5E8), medium (pCPA12) and low (QL9 and pCPC5), in 58α/β–/− cells transduced with CD8 and 42F3 TCR (Fig. 2b).

Table 1  Signaling and binding properties of 42F3 TCR–reactive peptides

| Peptide   | Sequence      | EC50 (µM) | Emax (IL-2 pg/ml) | Kd (µM)* | koff (s−1) | koff (M−1s−1) |
|-----------|---------------|-----------|-------------------|----------|-------------|---------------|
| QL9       | QLSPFPFDL     | 0.0447    | 399               | 40       | >5.00 × 10−1 | ND            |
| p4B10     | QLSVPMDL      | 0.000164  | 3,297             | 10       | >5.00 × 10−1 | ND            |
| p5E8      | FLSFWFDI      | 1.38      | 3,349             | 48       | >5.00 × 10−1 | ND            |
| pCPA12    | VPYMAEGFM     | 5.95      | 1,149             | 2        | 2.30 × 10−1  | 3.14 × 104    |
| pCPC5     | SPARPLDL      | 0.416     | 363               | 1        | 1.97 × 10−1  | 2.70 × 104    |
| pCPE3     | MPAQRFWDL     | >100      | ND                | 0.7      | 1.66 × 10−1  | 5.86 × 104    |
| pCPB7     | WPAGGQFDL     | 0.00286   | 2,844             | 0.1      | 5.79 × 10−2  | 4.95 × 105    |
| pCPB9     | SPAAGFSDL     | 0.07      | 3,144             | 0.1      | 6.68 × 10−2  | 2.40 × 105    |
| p3A1      | SPLDSLWLI     | Inactive  | Inactive          | 4        | 7.52 × 10−1  | 2.01 × 104    |

Kd, dissociation constant; koff, off rate; kon, on rate; ND, not determined.

*Values obtained from steady-state experiment.

Peptide degeneracy of the 42F3 TCR

We crystallized five 42F3 TCR complexes bound to a mini-H-2Ld scaffold (m31r) presenting the newly identified peptides (Fig. 3b, Supplementary Fig. 4a,b and Supplementary Table 1) and compared those structures with the structures of ‘wild-type’ QL9 (Fig. 3a) and four previously characterized 42F3 TCR–pMHC complexes5 (Fig. 3c and Supplementary Fig. 4c,d). The refolded m31r variant lacked the engineered linkers, circular permutation and synthetically evolved ‘stabilizing’ substitutions, which allowed us to study structures more representative of ‘naturally’ presented peptide, as we tested for activity (Fig. 2). Notably, every selected peptide assayed was recognized by the 42F3 TCR in both presentation formats (i.e., covalently linked in the selection scaffold, and as free peptide refolded with MHC) (Figs. 2 and 3). For each structure solved, the peptide backbone and side-chain electron density were well defined (Supplementary Fig. 4).

To visualize the adaptive molecular determinants of peptide cross-reactivity by the 42F3 TCR CDR3 loops, we comprehensively compared the CDR3-peptide contacts of both the first-generation complexes and the second-generation complexes (Fig. 3b,c). Each peptide was recognized by a unique pattern of pair-wise contacts with the TCR CDR3 loops (Fig. 3b,c). Generally, the CDR3β loop contributed more to peptide recognition than the CDR3α loop, probably due to the arch in the peptide at residues lying underneath CDR3β (Fig. 3b,c). In contrast, the CDR3α contacts

Figure 3  Peptide specificity of the 42F3 TCR. (a) Overall structure of the 42F3 TCR–QL9–H-2Ld complex: green, MHC; red, TCRα; blue, TCRβ; yellow, peptide. (b,c) Recognition of peptide by CDR3α and CDR3β loops of the 42F3 TCR for five (b) or four (c) synthetic peptides derived from the second-generation (b) or first-generation library (c) yeast-displayed peptide library, presented as structures, with ‘sticks’ indicating TCR residues making contacts to the peptides (left), and as contact maps (right): black lines, van der Waals contacts; red dashed lines, hydrogen bonds; blue arrows (TCR-exposed) and green arrows (MHC-buried) indicate direction of amino acid side chains on the TCR-pMHC complex.
were in general fewer and more diverse in each complex, consistent with the lack of sequence specificity at peptide positions P1–P4 (Figs. 1d and 3b, c). Overall, it seemed that the arch in the carboxy-terminal region of the peptide enforced close contact with CDR3β of the 42F3 TCR, while the ‘lower-lying’ amino-terminal peptide residues were more distant from CDR3α and permitted more sequence diversity in this region. In two complexes (with QL9 and pCPB7), CDR3α did not make contact with the peptide1 (Fig. 3b, c). Peptide conformation had a critical role in establishing contacts, as the peptide backbone, rather than its side chains, were typically recognized by the 42F3 TCR (Fig. 3b, c). For example, Lys95α of the 42F3 TCR frequently contacted the amide backbone of the peptide presented (for five of nine complexes), while Asp95β of the 42F3 TCR frequently formed a hydrogen bond through its backbone carbonyl to the amide of position P8 (for four of nine complexes) (Fig. 3b, c).

The most consistent set of van der Waals contacts within this set of complexes arose from hydrophobic residues at peptide position P7 to the Asp95-Ala96-Pro97 motif of the CDRβ3 loop (Fig. 3b, c). Since Pro97 restricted the dihedral angles of the loop, the CDRβ3 remained fixed in conformation in all nine complexes1 (Fig. 3b, c).
Other peptide positions whose sequences were biased in our enriched peptide pools (Fig. 1d) did not result in conserved contacts to the 42F3 TCR (Fig. 3b,c). For example, the ‘preferred’ acidic amino acid at P4 correlated with potent IL-2 responses but failed to show conserved contacts across these complexes (Fig. 3b,c). In the complexes with pCPB9 or p4B10, the acidic P4 side chain formed hydrogen bonds to Ser99α or Gly96α, respectively, of the 42F3 TCR. (Fig. 3b,c). In the complex with pCPB7, the Glu4 position did not seem to make contact with the CDR3α loop (Fig. 3b,c). Similarly, while our structural set included five complexes with peptides with the ‘preferred’ aspartic acid at P8, the contacts made by this residue varied from hydrogen bonding to van der Waals contacts to no contact with the TCR (Fig. 3b,c). Although we observed some amino acid ‘preferences’ at positions P4–P6 for recognition by the 42F3 TCR (Fig. 1c,d), only the ‘preferred’ hydrophobic amino acid at P7 resulted in conserved TCR-peptide interactions in agonist complexes (Fig. 3b,c).

Germline recognition in stimulatory receptor geometries

We next assessed how the TCR-MHC docking topology was modulated by the diverse agonist peptides derived from our selections. Combined, the nine complexes with the 42F3 TCR (one non-agonist and eight agonist) produced three main docking topologies at angles of 84°, 64° and 27° (Fig. 4a). The 27° angle noted for a non-agonist peptide has been characterized1, so here we focused on the eight agonist peptides.

The most frequent docking mode, including each of the CD8-dependent agonist complexes (QL9, p5E8, pCPA12, pCPC5 and pCPE3) and partial agonist complexes (QL9, pCPA12, pCPC5 and pCPE3), was 64° relative to the peptide (Fig. 4a). Notably, two potent agonist peptide–MHC complexes assumed an alternative docking geometry with a similar Vβ3.3 contact but a shifted Vα8.3 footprint, which rotated the TCR to ~84° (Figs. 3b and 4a). These two peptides (pCPB7 (WPAGGGQL) and pCPB9 (SPAAGGFL)) contained six identical residues among the nine total residues, including a glutamic acid at P4 and a glycine at P6. Residues at these positions differed from those in the peptides that exhibited the 64° docking angle (Figs. 3 and 4a). One possible explanation for this might be that the presence of a glycine at P6 provided sufficient flexibility of the peptide backbone to allow the TCR to pivot by 20°. Nevertheless, the slippage of the TCR-pMHC geometry from the 64° docking angle to the 84° docking angle did not seem to affect the maximal stimulatory potential of the TCR-pMHC complexes, as agonists with 84° TCR-docking geometries elicited IL-2 responses equivalent to that of the strongest agonist peptide with a 64° docking angle: p4B10 (Figs. 2 and 4a). We therefore observed three peptide-induced docking modes by which the 42F3 TCR was able to engage H-2Ld, two of which were able to maximally stimulate IL-2 responses and one (previously reported) that failed to stimulate 42F3 T cells1.

Notably, all agonist complexes, including published complexes of the 2C TCR with H-2Ld (refs. 12, 37), shared a recognition motif that was nearly completely atomically superimposable, mediated through the Vα3.3 domain, in which Tyr31α and Tyr50α of the 42F3 TCR buried Tyr155 of the MHC, and Ser51α of the 42F3 TCR formed hydrogen bonds to the Glu154 backbone (Fig. 4b and Supplementary Fig. 5a). The overall root-mean-square deviation of these three residues ranged from 0.9Å to 2.2Å compared with that of the 42F3–QL9–H-2Ld TCR-pMHC complex (data not shown). In contrast, the Vβ positions formed two groups, at a docking angle of either 64° or 84° (Fig. 4a). For the docking angle of 64°, 42F3 TCR Vβ consistently used Asn30β, Tyr50β and Ala52β to interact with the H-2Ldα1 helix at Glu72, Trp73, Ala76 and Arg79 (Supplementary Fig. 5b). For the docking angle of 84°, 42F3 TCR Vβ used Gln31β, Tyr50β and Gln71β to contact Gln65, Lys68, Gln72, Val76 and Arg79 of the H-2Ldα1 helix (Supplementary Fig. 5b). We therefore observed a pivot mechanism that facilitated peptide cross-reactivity in which two Vβ motifs for contact with the MHC accommodated diverse agonistic peptides, while the dominant Vα3 germline contacts were conserved across all agonist complexes studied for 2C, 2C variants and 42F3 TCRs recognizing H-2Ld (Fig. 4c and Supplementary Table 2).

DISCUSSION

The data we have presented here, together with published structures of the 42F3 and 2C TCRs and their variants bound to H-2Ld–QL9 (refs. 1,12,37), constitute a structural database of 12 TCRs bearing a common germline element (Vα3) and engaging a common MHC molecule (H-2Ld). This collection of 12 TCR-pMHC complexes is reflective of the general features of most TCR-pMHC interactions, as seen in the database of more than 50 published TCR-pMHC–MHC class I and TCR-pMHC–MHC class II structures. Unquestionably, given the extremely large combinatorial diversity of the TCR repertoire, deviations from these generalizations can be expected. Nevertheless, several conclusions about TCR cross-reactivity and germline recognition have emerged from our analysis here that are in accord with the majority of the overall structural database.

In terms of TCR cross-reactivity, we found that the 42F3 TCR did indeed recognize a wide range of peptides with limited homology to the cognate peptide QL9. However, while this would appear to support the concept that the TCR promiscuously recognizes many diverse peptides in a biological milieu31,32,38–41, close inspection of the chemistry in the TCR-pMHC interfaces revealed a more nuanced reality. Despite sequence variability, the 42F3 TCR repeatedly focused on structurally and chemically similar elements of the peptides, most commonly using the CDR3β Asp–Ala-Pro motif to engage ‘preferred’ hydrophobic residues at position P7. Recognition by the 42F3 TCR was therefore highly focused on a single apical peptide residue, with a wide range of chemistries and conformations used to accommodate the diversity at the remaining accessible positions. The limited interaction of the 42F3 TCR with the peptide’s amino terminus relaxed overall peptide specificity, which allowed recovery of a larger number of peptide sequences. It is possible, in fact it is likely, that an H-2Ld library with varied peptide lengths or MHC anchor residues could be used to identify peptides with substantially different TCR-binding solutions. However, we believe that our results reflect the general properties of most TCR-pMHC interactions. The scope and nature of cross-reactivity is consistent with published data focused on the recognition of MHC class II molecules by TCRs30. Collectively, these data suggest that TCRs are more specific than previously appreciated and lead to a more granular definition of cross-reactivity as being rooted in highly specific peptide hot spots that enable relaxed specificities at ancillary positions.

The conclusions of these studies reflect a fundamental feature of protein–protein interfaces: engagement of a small number of structurally and energetically important hot spots, typically near the interface center, surrounded by weaker and more diverse peripheral interactions17,30,42. Inspection of the amino acid sequences of peptides recognized by the 42F3 TCR in the absence of structural information would suggest degeneracy, while in fact recognition is focused on key features shared by recognized peptides. Although CDR3-peptide hot spots in TCR-pMHC interfaces have been discussed17,43, the observation that the same hot spot was repeatedly engaged in the 42F3 system, together with visualization of how sequence diversity was tolerated, allows better understanding of the nature of TCR cross-reactivity. This mechanism ensures the ability to engage large numbers...
of diverse peptides with retention of specificity for at least one structural and chemically homologous position. We suggest that parsing of the TCR-pMHC interface into hot-spot residues and non-hot-spot residues contributes substantially to the dichotomy of cross-reactivity and specificity that characterizes TCR recognition.2–10

With respect to germline recognition, we observed a striking, near-atomical superimposition of the interaction between Vα3.3 and the H-2Ld α2 helix in 12 structures with different peptides, Vβ segments and CDR3 loops. The repeated observation of this interaction, in the presence of considerable local and global structural variation, was probably reflective of the ‘imprint’ of TCR-MHC co-evolution, in this case a favorable ‘patch’ between Vα3.3 and H-2Ld. For the 42F3 TCR, these interactions seemed to dominate over the Vβ interactions, which permitted the observed ratcheting of Vβ along the α1 helix (dominant roles for α-chains in TCR binding have been observed before44). The inherent adaptability or ‘give’ available to TCR-MHC germline interactions is illustrated by the non-agonist peptide p3A1, whose Trp-Trp motif occludes the ‘preferred’ docking site for Vβ3.3, which results in a ‘peptide-centric geometry’1. Similar circumstances might be found with long or otherwise unusually ‘bulged’ peptides22,45.

Our observations suggest an interplay between TCR-MHC germline bias and its potential influence on cross-reactivity. It has been shown that alterations in TCR-docking modes can facilitate cross-reactivity,11–13,33 and that CDR3 loops can affect TCR-MHC contacts through ‘CDR3 editing’46. Consistent with published results,1,33,35 we also observed that different peptides yielded different binding modes even with the same TCR and MHC. However, rather than seeing the spectrum of binding topologies that would be expected from a purely ‘opportunistic’ system, we observed distinct, discrete binding modes for peptides in the form of the ratcheting of 42F3 TCR Vβ along the H-2Ld α1 helix or, equivalently, ‘preferences’ of the 42F3 TCR for particular binding orientations. The existence of these structural ‘preferences’, even in the case of the alloreactive TCR-MHC pair of the 42F3 TCR and H-2Ld that did not encounter each other during T cell development, supports the proposal of co-evolution of TCR and MHC. However, notably, these observations also support the proposal of the presence of a genetically encoded adaptability that can facilitate cross-reactivity. In this case, the orientations of 42F3 TCR Vβ probably stemmed from a combination of germline-encoded attractive interactions and repulsive interactions at ‘preferred’ orientations and ‘non-preferred’ orientations, respectively. From these data, we propose a model for engagement by the TCR that integrates germline bias and peptide recognition. TCRs are clearly adaptable, even capable of engaging in non-productive binding modes and, under certain circumstances, of binding non-MHC ligands.1,28,29,47.

We suggest the existence of ‘preferred’ but weak Vα or Vβ interaction points along MHC helices that bias TCRs toward, but do not obligate, discrete binding solutions. Chemically, this could arise from the incorporation of multi-functional amino acids such as tyrosine and the strategic placement of charges or hydrogen-bond donors and acceptors, as seen with the interaction points between the 42F3 TCR and H-2Ld. The resulting structural biases would help to focus binding while still permitting structural adaptations as the CDR3 loops accommodate peptide hot spots. In this way, the mechanisms of TCR cross-reactivity and germline bias are cooperative processes in shaping the recognition of pMHC complexes.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Protein Data Bank: coordinates and structure factors, 4MS8, 4MVB, 4MXQ, 4N0C and 4N5E.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank S. O’Herrin (Benn May Institute for Cancer Research) for the 58C12 ‘β’ cell line; J.M. Connolly (Washington University School of Medicine) for the 6M1.8-H-2LdW97R cell line; and N. Goriatcheva, E. Özkän, D. Wittrup, E. Newell, N. Jarvie and M. McLaughlin for discussions. Supported by the Canadian Institutes of Health Research (J.J.A.), the National Science Foundation (M.E.B.), the US National Institutes of Health (AI103867, AI045757 and AI057292 to K.C.G., and GM55767 to D.C.W.), the Jordan family, and the Howard Hughes Medical Institute (K.C.G.). Use of the Stanford Synchrotron Radiation Lightsource, SLAC National Accelerator Laboratory, is supported by the US Department of Energy, Office of Science, Office of Basic Energy Sciences under contract number DE-AC02-76SF00515. The SSRL Structural Molecular Biology Program is supported by the Department of Energy Office of Biological and Environmental Research and by the US National Institutes of Health, National Institute of General Medical Sciences (including P41GM103393).

AUTHOR CONTRIBUTIONS

J.J.A., S.N., M.E.B., D.M.K. and K.C.G. conceived of the project; J.J.A., S.N., and M.E.B. performed experiments and analyzed data; and all authors interpreted data, developed the concepts in the manuscript, and wrote and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Design and selection of H-2Ld-m31r-CP. To recover larger arrays of peptide from yeast selections, we optimized several features of the original yeast display scaffold. Of the five substitutions, three (N30D, A49D and L131R) arose distal to the TCR and peptide-binding surfaces; however, two substitutions arose in the bottom of the peptide-binding groove (W97R) or on the surface of the α1 helix (I66V).

To produce a closer mimic of H-2Ld for display, we reverted the peptide-proximal substitutions back to wild-type (Ile66 and Trp97) or via a 12-αmino acid glycine-serine (GGGGS) linker, and then the carbonyl terminus of the H-2Ld α2 domain to the amino terminus of the H-2Ld α1 domain (amino acids 1–120) via a six-α-mino acid glycine-serine linker. The construct was cloned into the vector pYAL via the Nhel and HindIII restriction sites. Yeast expressing this construct stained with antibody to the Myc epitope (9B11; Cell Signaling) but did not stain with 42F3 TCR tetrarmers or 2C TCR tetrarmers.

For selection of a functional variant of m31r-CP, variants of QL9-tethered scaffolds were amplified from 50 ng m31r-CP template DNA with a GeneMorph II Random Mutagenesis kit and were co-transformed into EBY100 at a vector/insert ratio of 1:5:5 µg, as described. Yeast tilters indicated that the error-prone library contained >1 x 10⁶ transformants. Selection was carried out by magnetic bead selection strategy.

The selections for a functional variant of m31r-CP were conducted with both 2C TCRs and 42F3 TCRs to ensure the library did not converge to a TCR-specific MHC construct. Induced yeast libraries were negatively selected with 500 µl anti-phycocerythrin magnetic beads (Miltenyi) with an LS column (Miltenyi) before tetramer positive selection. Positive selections were carried out with preformed 2C TCR tetrarmers at a concentration of 500 nM in PBS plus 0.5% BSA (PBS-BSA), with gentle mixture for 2.5 h at 4 °C. Cells were washed three times with PBS-BSA and were mixed with 500 µl anti-phycocerythrin magnetic beads (Miltenyi) in 5 ml PBS-BSA. Beads were gently mixed for 20 min at 4 °C, were washed twice for removal of unbound beads and were added to an LS magnetic column (Miltenyi). After being washed with three column volumes of PBS-BSA, the column was then removed from the magnetic field and bound cells were eluted in 5 ml of PBS-BSA. From the eluted yeast, 50 µl of cells were put aside for flow cytometry and counting of cell on a C6 flow cytometer (Accuri). The cells were then recovered over-night at 30 °C in SDCAA medium before dilution into 5 ml SGCAA induction medium. Cells were cultured for 48 h at 20 °C before the selection process was repeated. For all subsequent rounds of selections, ten times the number of eluted cells from the previous round were negatively selected with anti-phycocerythrin magnetic beads and were positively selected with 500 nM 2C or 42F3 TCR tetrarmers and 50 µl of anti-phycocerythrin magnetic beads in a volume of 1 ml. Populations underwent enrichment until enrichment of >10% TCR-tetramer staining within the co-selected yeast population, a final ‘polishing’ round of enrichment was carried out with multimeric 42F3 TCR. Individual clones were analyzed for enrichment of 100 nM TCR-tetramer by flow cytometry and were sequenced as described.

Protein expression, purification, and crystallization. For selections, biotinylated 2C and 42F3 TCRs were produced and biotinylated by co-expression of TCRα and TCRβ viruses in High Five Trichoplusia ni insect cells. The α- and β-chains were co-secreted with BirA ligase into medium in the presence of the medium Insect-XPRESS (Lonza) supplemented with 100 µM biotin, as described.

The 42F3 TCR was purified and refolded from E. coli inclusion bodies as described. An E. coli-expressed m31r refold construct was created by generation of the substitutions R97W and V66I in the H-2Ld mini-MHC protein already described. Peptides were synthesized (GenScript) and refolded at a concentration 20 µM in the presence of 300 mg m31r, as described. After dialysis, pMHC were precipitated with 50% saturation of ammonium sulfate at 4 °C and were purified by size exclusion on a Superdex 200 in HEPEs-buffered saline. The absorbance at 280 nm was used to determine the concentration of TCR and pMHC, and proteins were concentrated to 150 µM, at a molar ratio of 1:1, for crystal screening.

All crystals were formed in sitting drops by a vapor-diffusion method. The 42F3-pCPA12-m31r complex was crystallized in 16% PEG 3350, 100 mM TRIS, pH 7.5, and 200 mM magnesium nitrate and was cryogenically frozen in liquid nitrogen in crystallization buffer with 20% ethylene glycol. The 42F3-pCPB7-m31r complex was crystallized in 25% PEG 3350, 100 mM BIS-TRIS, pH 5.5, and 200 mM ammonium acetate and was cryogenically frozen in liquid nitrogen in crystallization buffer with 10% ethylene glycol. The 42F3-pCPB7-m31r complex was crystallized in 20% PEG 4000, 100 mM TRIS, pH 6.0, 200 mM sodium chloride and was cryogenically frozen in liquid nitrogen in crystallization buffer with 15% ethylene glycol. The 42F3-pCPB7-m31r complex was crystallized in 14% PEG 3350, 100 mM BIS-TRIS, pH 7.0, and 200 mM magnesium nitrate and was cryogenically frozen in liquid nitrogen in crystallization buffer with 20% ethylene glycol. The 42F3-pCPB3-m31r complex was crystallized in 17% PEG 3350 and 100 mM BIS-TRIS, pH 6.6, and 200 mM magnesium nitrate and was cryogenically frozen in liquid nitrogen in crystallization buffer with 20% ethylene glycol.

All crystallographic data were collected at the Stanford Synchrotron Radiation Lightsource (Stanford) beamline 12-2. Data sets were indexed and scaled with the MOSFLM diffraction data-integration program and the SCALA scaling and data-merging program, except for the pCPB7 construct, which was indexed and scaled with HKL-2000 program suite. All data sets were phased by molecular replacement with the Phaser program for phasing macromolecular crystal structures by maximum-likelihood methods through searching for TCR and pMHC independently (Protein Data Bank accession code, 3TFK), then were refined with the PHENIX software suite and were modified with the COOT program. Structural analysis, alignments and image rendering were carried out with the PyMOL molecular graphics system.

T cell stimulation. All peptides were diluted to 50 nM in DMSO. LM1.8 cells transduced with H-2Ld-W97R (described below) were grown to a confluency of 90% in T75 flasks, in RPMI medium with 10% FBS plus penicillin, streptomycin and γ-glutamine. T cells were stimulated as described. LM1.8 cells (5 x 10⁵) transduced with H-2Ld-W97R and peptides diluted in RPMI medium were incubated for 20 h at 37 °C at a ratio of 1:1 (effector cell/target cell) with CD8αβ or CD8αβ⁺ 58αβ⁻/β⁻ lines transduced to express the 42F3
TCR. Supernatants were analyzed for release of IL-2 by horseradish peroxidase colorimetric enzyme-linked immunosorbent assay (BD Pharmingen).

The 42F3 TCR–transduced cell lines were generated as described with the T cell hybridoma cell line 58α−/β− with and without CD8αβ. The 58α−/β− cell line was provided to the laboratory of D.M. Kranz by S. O’Herrin. These cell lines have not been tested for mycoplasma contamination; however, no contamination was observed during the generation of these cell lines or in their subsequent culture and maintenance.

The antigen-presenting cell line used in this study, LM1.8 cells transduced with H-2LdW97R, is an L cell mouse fibroblast cell line provided by J.M. Connolly. This cell line was not tested for mycoplasma contamination during its use in the laboratory of D.M. Kranz; however, no contamination was observed during the culture or maintenance of this cell line.

The antibodies used in T cell–activation studies included anti–mouse CD3ε (145-2C11; BD Pharmingen), purified rat anti–mouse IL-2 (JES6-1A12; BD Pharmingen) and biotinylated rat anti–mouse IL-2 (JES6-SH4; BD Pharmingen). Antibody to mouse TCR constant-region β-chain (H57-597; BD Pharmingen) was used for detection of expression of the 42F3 TCR on the surface of 42F3 TCR–transduced 58α−/β− cells during generation of the cell line created via retroviral transduction.

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