A highly tilted binding mode by a self-reactive T cell receptor results in altered engagement of peptide and MHC

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Self-reactive lymphocytes that escape elimination in the thymus can cause autoimmune pathology, and it is therefore important to understand the structural mechanisms of self-antigen recognition. We report the crystal structure of a T cell receptor (TCR) from a patient with relapsing–remitting multiple sclerosis that engages its self–peptide–major histocompatibility complex (pMHC) ligand in an unusual manner. The TCR is bound in a highly tilted orientation that prevents interaction of the TCR-α chain with the MHC class II β chain helix. In this structure, only a single germline-encoded TCR loop engages the MHC protein, whereas in most other TCR–pMHC structures all four germline-encoded TCR loops bind to the MHC helices. The tilted binding mode also prevents peptide contacts by the short complementarity-determining region (CDR) 3β loop, and interactions that contribute to peptide side chain specificity are focused on the CDR3α loop. This structure is the first example in which only a single germline-encoded TCR loop contacts the MHC helices. Furthermore, the reduced interaction surface with the peptide may facilitate TCR cross-reactivity. The structural alterations in the trimolecular complex are distinct from previously characterized self-reactive TCRs, indicating that there are multiple unusual ways for self-reactive TCRs to bind their pMHC ligand.

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it only partially occupied the binding groove. These results suggest that the observed structural defects in these trimolecular recognition units are caused by selection events that eliminated self-reactive T cells with optimal TCR binding properties. T cells that expressed the three EAE TCRs were pathogenic (Governan et al., 1993; Lafaille et al., 1994; Pearson et al., 1997), and transgenic mice that expressed the human Ob.1A12 TCR and the relevant human MHC molecule developed spontaneous CNS inflammation and demyelination (Madsen et al., 1999; Ellmerich et al., 2005). These TCRs thus have altered binding properties that apparently enabled escape from negative selection but still allowed them to bind to their target peptide–MHC (pMHC) complex with sufficient strength to cause disease. The vast majority of antimicrobial TCRs are positioned over the center of the pMHC surface. An N-terminal shift has so far been observed in one case, apparently also as a result of self-tolerance mechanisms (Gras et al., 2009). This TCR (CF34) was specific for an HLA-B8–bound EBV peptide and originated from a person who was HLA-B8 and HLA-B44 heterozygous. The N-terminal shift by this TCR appeared to prevent self-reactivity to HLA-B44. In contrast, the LC13 TCR was alloreactive for HLA-B44 because it was isolated from a person who expressed HLA-B8 but not HLA-B44. This TCR bound over the C-terminal part of the HLA-B8–bound EBV peptide (Gras et al., 2009).

However, the structural database is still too small to adequately describe the recognition properties of self-reactive T cells at a repertoire level, and a substantially larger number of complexes needs to be crystallized to determine to what extent self-reactive TCRs deviate from the rules followed by most antimicrobial TCRs. In particular, the range of possible binding solutions by human autoimmune T cells remains largely unexplored. A large number of crystal structures, as well as functional studies, have shown that antimicrobial TCRs use a diagonal binding mode that positions the four germline-encoded TCR loops (complementarity-determining region [CDR] 1 and CDR2 of TCR-α and -β) over the MHC helices (Sun et al., 1995; Garboczi et al., 1996; Garcia et al., 1996; Garcia and Adams, 2005; Rudolph et al., 2006; Marrack et al., 2008). In most structures, the CDR3 loops also contribute to MHC binding. This binding mode allows the two hypervariable CDR3 loops to make extensive contacts with the bound peptide. In addition, peptide contacts are frequently made by the CDR1 and CDR2 loops to N-terminal and C-terminal peptide residues, respectively. A substantial body of recent work has suggested that the observed placement of the germline-encoded CDR1 and CDR2 loops on the MHC helices is the product of coevolution of MHC and TCR genes (Turner et al., 2006; Feng et al., 2007; Dai et al., 2008; Garcia et al., 2009).

Previous studies on human autoimmune TCRs focused on two DR-restricted T cell clones specific for MBP from MS patients. We now report the crystal structure of a human TCR (Hy.1B11) from a relapsing-remitting MS patient that recognizes the same MBP peptide (residues 85–99) as Ob.1A12 TCR but presented by a different MHC molecule, HLA-DQ1 (abbreviated as DQ1). The T cell response to MBP in this patient was dominated by three in vivo expanded T cell clones specific for the MBP85–99 peptide that persisted over time. Two of these clones were DR restricted (DRA, DRB1*1602) and one was DQ1 restricted (DQAI*0102, DQB1*0502; Wucherpfennig et al., 1994a,b). A total of three clones with the Hy.1B11 TCR sequence (TRAV13–1*02, TRAJ48*01, and TRBV7–3*01, TRBD2*01, TRBJ2–3*01) were isolated from independent cultures, two from the initial time point and a third 13 mo later (Wucherpfennig et al., 1994b). Furthermore, this T cell clone was activated by four microbial peptides (from herpes simplex virus, adenovirus, human papillomavirus, and pseudomonas) that had limited sequence similarity to the MBP85–99 peptide (Wucherpfennig and Strominger, 1995). The structure showed that only one of the two CDR3 loops contacted the bound peptide, explaining why specificity was limited to few peptide residues. This self-reactive TCR thus followed some of the rules established for antimicrobial TCRs (such as binding of the TCR-β chain to the MHC α1 helix) while clearly violating other rules (no MHC helix contacts by TCR-α germline-encoded loops and peptide contacts by only one CDR3 loop). The structure thereby reveals a novel way of self-pMHC recognition by a TCR from a patient with a chronic inflammatory disease.

RESULTS

Unusual features of Hy.1B11 TCR binding to the self-pMHC complex

The complex of Hy.1B11 TCR and DQ1-MBP85–99 peptide crystallized in the space group P212121, and one of the crystals diffracted to a resolution of 2.55 Å. The structure was determined by molecular replacement with one molecule in the asymmetric unit and refined to Rwork/Rfree values of 23.2 and 25.8%, respectively. Crystal data and refinement statistics for the structure are shown in Table S1. Excellent electron density was observed at the interface for the CDR loops of the TCR, the peptide, and the DQ1 helices (Fig. S1). The structure showed a strong tilt in TCR binding toward the DQ1 α1 helix, which prevented interaction of the TCR Vα chain with the DQ1 β1 helix (Fig. 1, a, c, and e). The tilt was 14.5° compared with the influenza hemagglutinin (HA306–318)-specific HA1.7 TCR (Hennecke et al., 2000; Fig. 1, b, d, and f), measured using a vector through the centers of mass of the Vα and Vβ domains. In addition, a small crossing angle (40°) of the TCR over the pMHC surface was observed which apparently prevented MHC engagement by the CDR1B loop. This crossing angle was smaller than for any other studied MHC class II restricted TCR (Fig. S2), but a similar crossing angle had been observed for the MHC class I restricted 2C TCR (Garcia et al., 1998; Rudolph et al., 2006). As a result of the tilt and the small crossing angle, the center of mass of the Vα domain was shifted toward the peptide by ~6 Å compared with HA1.7, and the center of mass of the Vβ domain was also shifted by ~6.5 Å (Fig. S2). The tilted position resulted in limited Hy.1B11 TCR interaction with the DQ1 B1 helix (Table I) and also substantially reduced TCR interactions...
by the CDR3 loop because the CDR1 and CDR2 loops did not bind to DQ1.

Only a single germline-encoded Hy.1B11 TCR loop binds to HLA-DQ1

Four germline-encoded TCR loops, CDR1 and CDR2 of both TCR chains (Fig. 2 b, yellow), and both CDR3 loops (Fig. 2 b, red) typically contact the MHC helices, as illustrated for the human HA1.7 TCR (Hennecke et al., 2000). In stark contrast, only a single germline-encoded loop of Hy.1B11 TCR (CDR2β) bound to DQ1 (Fig. 2 a). There were no contacts between the TCR Vα domain and the DQ1β1 helix, and only a single residue on the DQ1β1 helix (E66) was contacted by Hy.1B11 TCR (through CDR3β; Fig. 2 e and Table I). Hy.1B11 TCR thus formed extensive interactions with the DQ1α1 helix but only limited contacts with the DQ1β1 helix (Fig. 2, c and e), whereas HA1.7 TCR had extensive interactions with both MHC helices (Fig. 2, d and f). The absence of MHC binding by three of the germline-encoded TCR loops was partially compensated by both CDR3 loops, in particular CDR3α, which made a substantial number of contacts with the DQ1α1 helix (Table I; Fig. 2, c and e).

Two distinctive features of Hy.1B11 TCR binding were responsible for this highly unusual interaction with the MHC molecule: the tilt that prevented DQ1 binding by the germline-encoded TCR-α chain loops and a small crossing angle.

Table 1. Contacts of Hy.1B11 TCR with HLA-DQ1

<table>
<thead>
<tr>
<th>CDR</th>
<th>TCR residue</th>
<th>DQ1 residue</th>
<th>Number of contacts</th>
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<tr>
<td>CDR3α</td>
<td>G96</td>
<td>Q57</td>
<td>2</td>
</tr>
<tr>
<td>CDR3α</td>
<td>G96</td>
<td>G58*</td>
<td>2</td>
</tr>
<tr>
<td>CDR3α</td>
<td>N97</td>
<td>D55</td>
<td>2</td>
</tr>
<tr>
<td>CDR3α</td>
<td>N97</td>
<td>Q57</td>
<td>3</td>
</tr>
<tr>
<td>CDR3α</td>
<td>E98</td>
<td>R61**</td>
<td>11</td>
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<td>CDR2β</td>
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<td>Q48</td>
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<tr>
<td>CDR3β</td>
<td>L95</td>
<td>E66</td>
<td>2</td>
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Altered self-pMHC engagement by an autoreactive TCR | Sethi et al.

Furthermore, mutation of this loop in TCR-β single chain transgenic mice interfered with T cell development (Scott-Browne et al., 2009). Comparison of Hy.1B11 TCR, two other human TCRs (HA1.7 and 3A6), and the mouse B3K506 TCR (as well as four other investigated TCRs; not depicted) showed a similar overall positioning of this CDR2β loop on the MHC class II α1 helix (Fig. 3, a–d; Marrack et al., 2008). Among all four TCRs, CDR2β residues 48 and 54 contacted the same positions on the MHC class II α1 helix (positions 61 and 57, respectively; Fig. 3 e), even though many of the interacting MHC and TCR side chains were chemically different. In addition, CDR2β residue 46 of Hy.1B11 and B3K506 TCRs interacted with residue 61 on the MHC α1 helix, whereas CDR2β residue 53 of both Hy.1B11 and 3A6 TCR bound to positions 57 and 61 on the MHC. Although the
overall positioning of the β2 loops on the MHC class II α1 helix was similar, the molecular details of the interactions were distinct as a result of differences in docking angles, MHC polymorphism, and the actual sequences of the TCR. CDR2β loops.

Only one of the CDR3 loops interacts with the MBP peptide

In most previously examined complexes of TCR and pMHC, both CDR3 loops contacted the bound peptide (as exemplified by HA1.7 TCR; Fig. 4 b). CDR3β apparently could not contact the DQ1-bound peptide because of its short length and the unusual tilt of Hy.1B11 TCR. The Hy.1B11 CDRβ3 loop was 10 amino acids in length compared with 9–16 amino acids in other TCR structures (Table S2). The majority of peptide contacts were instead made by the CDR3α loop, in particular F95α (TRAJ48), which was inserted deeply between the P2 His and P3 Phe side chains of the peptide (Fig. 4 a and Table II). Furthermore, the main chain carbonyl of F95α formed a hydrogen bond with P5 Lys of the peptide. Only one other residue of this CDR3 loop, E98α, contributed to peptide recognition through two contacts with P5 Lys. Functional experiments using a set of single amino acid analogue peptides confirmed that peptide specificity of the Hy.1B11 T cell clone was limited to the P2 to P5 peptide segment to which the CDR3α loop bound. All alanine analogues within this segment showed a substantial reduction in T cell proliferation (100 nM; Fig. 5 a). However, only the alanine substitution of P5 Lys resulted in a complete loss of activity (Fig. 5 b), sug-

Table II. Contacts of Hy.1B11 TCR with the MBP<sub>85-99</sub> peptide

<table>
<thead>
<tr>
<th>CDR TCR residue</th>
<th>Residue of peptide</th>
<th>Number of contacts</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDR1α</td>
<td>S28&lt;sup&gt;+&lt;/sup&gt;</td>
<td>P-3 Asn</td>
</tr>
<tr>
<td>CDR3α</td>
<td>F95&lt;sup&gt;-&lt;/sup&gt;</td>
<td>P2 His</td>
</tr>
<tr>
<td>CDR3α</td>
<td>F95</td>
<td>P3 Phe</td>
</tr>
<tr>
<td>CDR3α</td>
<td>F95&lt;sup&gt;-&lt;/sup&gt;</td>
<td>P5 Lys</td>
</tr>
<tr>
<td>CDR3α</td>
<td>E98</td>
<td>P5 Lys</td>
</tr>
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*Putative hydrogen bond to peptide sidechain (*) or to peptide backbone (#).*

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intermediate binding affinity of Hy.1B11 TCR

Surface plasmon resonance measurements were performed to compare the binding affinity of the MBP<sub>85-99</sub>-specific Hy.1B11 and Ob.1A12 TCRs for their ligands (DQ1-MBP<sub>85-99</sub> and DR2-MBP<sub>85-99</sub>, respectively). Monobiotinylated pMHC complexes were captured on a streptavidin sensor chip and soluble Hy.1B11 or Ob.1A12 TCR was injected over these surfaces. Hy.1B11 TCR bound to DQ1-MBP<sub>85-99</sub> with a K<sub>d</sub> of 14.3 µM based on equilibrium binding data (Fig. 6 b and Fig. 7 b). In contrast, the affinity of Ob.1A12 TCR for DR2-MBP<sub>85-99</sub> was lower, at ~100 µM (our measurements; Cole et. al., 2007). Hy.1B11 TCR binding was specific because a control TCR (Ob.1A12) showed no binding to DQ1-MBP<sub>85-99</sub>
the site of negative selection, whereas HLA-DR protein could be readily detected (Ishikura et al., 1987). It is also well known that HLA-DQ molecules are expressed at substantially lower (≈10-fold) than HLA-DR molecules on peripheral antigen-presenting cells (Roucard et al., 1996). Direct comparison of Hy.1B11 and Ob.1A12 T cell clones showed that Hy.1B11 T cells required higher concentrations of MBP85-99 peptide for stimulation, despite the higher affinity of Hy.1B11 TCR for its pMHC ligand (Wucherpfennig et al., 1994a). The higher affinity of Hy.1B11 compared with Ob.1A12 TCR may therefore compensate for the substantially lower level of HLA-DQ than HLA-DR expression.

Mutagenesis of MHC and peptide contacts

Given this unusual binding topology, alanine scanning mutagenesis was performed to validate the structure and to define the site of negative selection, whereas HLA-DR protein could be readily detected (Ishikura et al., 1987). It is also well known that HLA-DQ molecules are expressed at substantially lower levels (≈10-fold) than HLA-DR molecules on peripheral antigen-presenting cells (Roucard et al., 1996). Direct comparison of Hy.1B11 and Ob.1A12 T cell clones showed that Hy.1B11 T cells required higher concentrations of MBP85-99 peptide for stimulation, despite the higher affinity of Hy.1B11 TCR for its pMHC ligand (Wucherpfennig et al., 1994a). The higher affinity of Hy.1B11 compared with Ob.1A12 TCR may therefore compensate for the substantially lower level of HLA-DQ than HLA-DR expression.

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key TCR contact residues for DQ1 and the MBP85-99 peptide. All TCR residues that contacted DQ1 were mutated to alanine, except native alanine or glycine residues. In addition, CDR3α F95 was mutated to alanine, given its central role in peptide recognition (Fig. 7, a and b). For each mutant, nine concentrations were tested under equilibrium binding conditions to obtain reliable affinity measurements (Fig. S5). As a control, a double mutant of two noncontacting residues in CDR1α and CDR2α (N31A and S52A) was generated. This double mutant showed an affinity to DQ1-MBP85-99 close to wild-type Hy.1B11 TCR (13.8 and 14.3 µM, respectively). Four of the nine mutants had greatly reduced affinities for DQ1-MBP85-99, and these targeted TCR residues made contacts solely to DQ1 (CDR3β L95A, 110 µM), to both DQ1 and MBP85-99 peptide (CDR2β Q48A, 108 µM; and CDR3α E98A, >250 µM), or only the peptide (CDR3α F95A, >250 µM). These mutagenesis data also showed that the unusual TCR binding mode was not a crystallization artifact.

Binding by the CDR2β loop involved a combination of side chain and main chain contacts (Table I). The Q48A mutant showed substantially reduced DQ1-MBP85-99 binding (108 µM; Fig. 7 b). However, the binding contribution from two main chain hydrogen bonds (CDR2β Thr 50–DQ1α His 68 and CDR2β Ala 53–DQ1α Gln 57) could not be assessed with these mutants (Table I), which made it difficult to determine the total contribution of this TCR loop to DQ1 binding.

In contrast, CDR3α contacts were predominantly made by side chains. CDR3α E98A and F95A mutations resulted in a severe reduction in binding (>250 µM for both mutants). CDR3α F95 formed the majority of peptide contacts, and mutation to alanine greatly reduced TCR binding affinity for DQ1-MBP85-99. Nevertheless, this mutation did not assess the entire contribution by F95 because it did not eliminate the hydrogen bond between the main chain of F95 and the peptide side chain P5 Lys. The CDR3α E98A mutation resulted in the most substantial reduction in DQ1-MBP85-99 binding because of loss of two hydrogen bonds to DQ1α R61 and 11 other contacts, including two contacts to P5 Lys of the peptide.

CDR3β made no contacts to the peptide and only limited contact to the DQ1α helix. CDR3β L95 contacted DQ1β E66, and the L95A mutant showed a substantial reduction in affinity (110 µM). The interaction of CDR3β with the DQ1β helix also involved water-mediated hydrogen bonds. DQ1β E66 hydrogen bonded with a water molecule, which, in turn, hydrogen bonded with CDR3β main chain (S93, A94, L95, and E96) and side chain (D97) residues. It is possible that this hydrogen bonding network was destabilized by the CDR3β L95A mutation.

The mutagenesis data showed that all three TCR loops that contacted the DQ1 helices in the crystal structure contributed to DQ1 binding in this functional assay. The data also highlighted the critical energetic role of the CDR3α loop in binding to the DQ1-MBP85-99 complex, in particular the important contributions by CDR3α F95 and E98, which made many contacts to the peptide (CDR3α F95) and DQ1 (CDR3α E98) in the structure.

**Figure 7. TCR mutagenesis identifies key contact residues in the CDR2β and CDR3 loops.** (a) TCR point mutants are shown as colored balls: mutations in CDR2β in shades of green, CDR3α mutants in shades of red, and the CDR3β mutant in orange. Darker colors indicate stronger effects of mutations. pMHC is shown as a cartoon in silver. (b) Table showing the equilibrium binding affinity constants of TCR mutants. The number of contacts made to peptide or MHC by each residue are enumerated, along with the number of contacts disrupted by substitutions with alanine. Hydrogen bonds are indicated in parentheses. The equilibrium binding experiments were performed at least twice.

**DISCUSSION**

Autoaggressive T cells have to meet two competing requirements: they have to escape negative selection in the thymus, yet they need to be capable of initiating TCR signals of sufficient strength upon recognition of the self-antigen in the target organ of the disease. The prior structural characterization of two human TCRs from MS patients (Hahn et al., 2005; Li et al., 2005) and of three mouse TCRs from the EAE model (Maynard et al., 2005; Feng et al., 2007) showed in each case significant structural alterations that impaired TCR binding to the MHC-bound self-peptide (Wucherpfennig et al., 2009). The human TCRs bound with an altered topology, whereas the mouse TCRs bound with a normal topology. However, in all cases the interaction with the peptide was compromised either because of a shift in TCR binding (human TCRs) or partial occupancy of the peptide binding groove (murine TCRs). The structure of the self-reactive Hy.1B11 TCR
represents the first case in which only one of the four germline-encoded TCR loops interacts with the MHC helices. Furthermore, only one of the CDR3 loops interacts with the peptide, limiting TCR specificity to a short peptide segment (P2 His, P3 Phe, and P5 Lys). The structure thus explains how Hy.1B11 TCR responds to multiple microbial peptides that share limited sequence similarity with MBP<sub>85-99</sub> in particular P3 Phe, a basic residue at P5, and hydrophobic anchors at P1 and P4 (Fig. S6; Wucherpfennig and Strominger, 1995).

The Hy.1B11 and Ob.1A12 TCRs recognize the MBP<sub>85-99</sub> peptide bound in the same register to DQ1 and DR2, respectively (Fig. S3). The structures of the bound peptides are thus quite similar, except that the P4 Phe side chain is positioned deeper in the hydrophobic P4 pocket of DQ1 (Fig. S3 c). Interestingly, specificity of both TCRs is focused on the P2, P3, and P5 peptide sidechains, but through different structural mechanisms; Ob.1A12 TCR is shifted toward the peptide N terminus, which centers the CDR3 loops over P2 His, whereas Hy.1B11 is positioned over the center of the peptide binding groove but recognizes peptide side chains only through CDR3<sub>α</sub> and not CDR3<sub>β</sub>. It is possible that both TCRs bind to this peptide segment because it is more firmly anchored in the peptide binding groove, whereas the C-terminal part of the MBP<sub>85-99</sub> peptide is raised in both structures (Fig. S3 c; Smith et al., 1998). In both cases, the TCR contacts one of the MHC helices in the typical location, either the MHC class II α1 chain helix (Hy.1B11 TCR–β chain with CDR2<sub>β</sub> loop) or the MHC class II β1 helix (Ob.1A12 TCR–α chain with CDR1<sub>α</sub> and CDR2<sub>α</sub> loops; Fig. S7). However, the germline-encoded loops of the other TCR chain either do not engage the MHC molecule (Hy.1B11) or bind in a highly unusual location (Ob.1A12).

In the majority of previously determined structures, all four germline-encoded loops contact the MHC helices (21 of 29 structures, with each TCR counted only once using the structural database that is now available. The importance of the germline loops of both TCR chains in MHC restriction has been repeatedly demonstrated using mutagenesis approaches and analysis of natural MHC micropolymorphisms (Sim et al., 1996; Manning et al., 1998; Wu et al., 2002; Huseby et al., 2006). Recent structural and functional studies strongly support the hypothesis that the conventional diagonal TCR binding mode is the result of coevolution between MHC and TCR genes (Turner et al., 2006; Feng et al., 2007; Dai et al., 2008; Marrack et al., 2008; Garcia et al., 2009). The diagonal binding orientation on the pMHC surface is similar among most crystallized αβ TCRs, but because of variation in the binding angle it has not been possible to identify conserved MHC residues contacted by all TCRs (Baker and Wiley, 2001; Rudolph et al., 2006). However, seven crystal structures involving six different V<sub>β</sub>8.2 TCRs and one V<sub>β</sub>8.1 TCR that bound mouse I-A<sub>α</sub> molecules showed a close convergence of CDR1<sub>β</sub> and CDR2<sub>β</sub> contacts with the I-A<sub>α</sub> helix (Garcia et al., 2009). These results suggest that particular V<sub>β</sub> domains have preferred binding sites on the MHC helices. In mice with a single rearranged TCR–β chain, mutation of the CDR2<sub>β</sub> residues Tyr<sub>46</sub> or Tyr<sub>48</sub> to peptides bulging out of the MHC class I binding groove. A super-bulged 13-aminopeptide bound to HLA-B<sup>∗</sup>3508 is recognized by SB27 TCR and the interface is dominated by TCR–peptide interactions. Nevertheless, each of the four germline-encoded TCR loops contacts the MHC protein, even though these interactions are limited (Tyan et al., 2005). In another example, the ELS4 TCR flattens a peptide bulging out of the groove of HLA-B<sup>∗</sup>3501, which enables more extensive MHC contacts. Again, all four germline-encoded loops are involved in MHC binding (Tyan et al., 2007). The BM3.3 TCR shows cross-reactivity between V<sub>SB</sub>V8 and pBMI peptides bound to H-2K<sup>β</sup>, and there are large differences in the contribution of the V<sub>α</sub> and V<sub>β</sub> chains to the interface between the two structures (Reiser et al., 2000). Nevertheless, all four germline-encoded TCR loops contribute to MHC recognition in both complexes.

The YAe62 TCR was isolated from mice in which negative selection was severely limited by expression of a single MHC class II–peptide complex in the thymus (Huseby et al., 2005). Even though YAe62 TCR contacts both MHC helices, it has a substantially larger interaction surface with the α1 than the β1 helix of the MHC molecule because of a tilt in TCR binding (Dai et al., 2008). Unbalanced TCR interactions with the two MHC helices can thus occur in T cells that were either not subjected to negative selection (YAe62 TCR) or escaped elimination in the thymus (Hy.1B11 TCR). Furthermore, there are now three TCRs that recognize the bound peptide using only one CDR3 loop: YAe62 and BM3.3 TCRs with their CDR3<sub>β</sub> loops and Hy.1B11 with its CDR3<sub>α</sub> loop (Reiser et al., 2000; Dai et al., 2008). The YAe62 TCR is extremely cross-reactive to both peptide and MHC variants, and the BM3.3 TCR was shown to cross react with a viral octapeptide presented by H-2K<sup>β</sup>. Peptide recognition by a single CDR3 loop is therefore also unusual within the large structural database that is now available.

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alanine caused a substantial reduction in the number of thymocytes. Furthermore, these mutations changed Vα usage, which is apparently a compensatory mechanism to enable positive selection of some T cells despite reduced MHC binding by the Vβ chain (Scott-Browne et al., 2009). Studies on MHC class I restricted TCRs have also identified MHC residues that are frequently recognized by TCRs, but the contribution of these MHC residues to binding differs among individual TCRs (Burrows et al., 2010).

CDR2β residues of Hy.1B11 were found to interact with a similar set of MHC positions as other MHC class II restricted TCRs. Given that CDR2β is the only germline-encoded TCR loop that contacts DQ1, it is possible that the placement of this loop on the DQ1 α1 helix contributed to the diagonal position of this TCR over the center of the DQ1-MBP85-99 surface. Alanine scanning mutagenesis showed that the two CDR3 loops contributed significantly to DQ1 binding. CDR3 loops have been shown to be flexible (Garcia et al., 1998; Hare et al., 1999; Armstrong et al., 2008) and, in addition to contacting the peptide, they can make important contributions to MHC binding (Garbozzi et al., 1996; Borg et al., 2005). The orientation of Hy.1B11 TCR on the DQ1-MBP85-99 surface therefore appears to result from the combined binding contributions of the CDR2β and both CDR3 loops.

The unusual structure of the Hy.1B11 TCR–DQ1-MBP85-99 complex also raises the important question of how the corresponding human T cell escaped from negative selection in the thymus, but caution has to be used to extrapolate from structural data to complex in vivo events. This TCR has a higher affinity for its pMHC target than the previously crystallized self-reactive TCRs Ob.1A12 and 3A6 (Hahn et al., 2005; Li et al., 2005). However, the Hy.1B11 TCR is HLA-DQ restricted, whereas the other two TCRs are HLA-DR restricted. HLA-DQ molecules are expressed at the thymus, but caution has to be used to extrapolate from structural data to complex in vivo events. This TCR has a higher affinity for its pMHC target than the previously crystallized self-reactive TCRs Ob.1A12 and 3A6 (Hahn et al., 2005; Li et al., 2005). However, the Hy.1B11 TCR is HLA-DQ restricted, whereas the other two TCRs are HLA-DR restricted. HLA-DQ molecules are expressed at the thymus, but caution has to be used to extrapolate from structural data to complex in vivo events.

**MATERIALS AND METHODS**

Protein expression and complex formation. Hy.1B11 TCR used the gene segments TRAV13-1*02, TRAJ48*01 (non-nucleotide–encoded sequence, g), CDRα3 protein sequence, AASSFGNEKET and TRBV7-3*01, TRBD2*01, TRBJ2-3*01 (non-nucleotide–encoded sequence, cctgagcct; CD83 protein sequence, ATSALGDTQY). In the expression construct, the MBP85-99 peptide was attached to the N terminus of the TCR–β chain through a flexible octapeptide linker (GGSGGGGSG), as reported by Hennecque et al. (2000). The interchain disulfide bond located at the C terminus of the Cα and Cβ Ig domains was moved to the N-terminal part of these domains (replacement of Cα Thr48 and Cβ Ser57 with cysteines) to enhance refolding of TCR heterodimer (Boulter et al., 2003). The chains were separately cloned into pET-22b vector (Novagen), and inclusion bodies produced in BL21(DE3) E. coli cells (Novagen) were dissolved in 6 M guanidine hydrochloride, 10 mM dithiothreitol, and 10 mM EDTA. To initiate refolding, TCR-α- and β-chains were diluted at a 1:1 molar ratio to a concentration of 25 μM of each chain in a refolding buffer containing 4.5 M urea, 0.5 M t-arginine–HCl, 100 mM Tris-HCl, pH 8.2, 1 mM of reduced glutathione (GSH), and 0.1 mM of oxidized glutathione (GSSH). After 40 h at 24°C, the refolding mixture was dialyzed twice against denatured water and twice against 10 mM Tris-HCl, pH 8.0. Refolded TCR was purified by anion exchange chromatography using Poros PI (Applied Biosystems) and MonoQ (GE Healthcare) columns. TCR mutants were generated by overlapping PCR and cloned into the pET-22b vector (Novagen). These mutant proteins were refolded and purified using the same procedure as wild-type TCR Hy.1B11.

DQ1 was produced in glycosylation-deficient Lec3.2.8.1 cells (Stanley, 1989). The CLIP peptide was attached to the N terminus of the DQ1β chain using a linker with a thrombin cleavage site, and the two chains were cloned into a vector that drives expression of glutamine synthetase to enable secretion of transfected clones in glutamine-deficient media (Day et al., 2003). Stable clones were produced under methionine sulphoximine selection and tested for DQ1 secretion by Western blotting. The clone with the highest DQ1 production level was expanded in a hollow fiber bioreactor (AccuSys miniMax; Biovert International) and secreted DQ1 was affinity-purified using mAb 9.3.1 (American Type Culture Collection), Fos and Jun leucine zipper dimerization domains at the C terminus used to facilitate DQ1 heterodimer formation were removed by V8 protease cleavage.

After cleavage of the CLIP peptide linker, complexes were formed by permitting binding of the TCR–β chain–linked MBP85-99 peptide to the DQ1 binding site. TCR, DQ1, and HLA-DM were incubated at a molar ratio of 6:4:1 for 18 h at 25°C at a pH of 5.4, and the complex was separated from components by gel filtration (Superdex S-200 column; GE Healthcare) and anion-exchange chromatography (MonoQ; GE Healthcare).

Crystallization and data collection. The complex was determined to be pure by SDS-PAGE and isoelectric focusing PAGE. The complex was concentrated to 7.5 mg/ml in 10 mM Hepes, pH 7.2. A crystallization matrix based on conditions in which other TCR–MHC complex crystals were obtained was used as a starting point for screening. Crystals were obtained in multiple conditions. The final crystals for data collection were grown by the hanging-droplet vapor-diffusion method against a reservoir of 0.1 M ammonium sulfate, 8–10% PEG 8000, and 50 mM sodium citrate, pH 6.1, at 24°C. Crystals were cryoprotected by the addition of ethylene glycol to 25%. Data were collected at 100 K at the National Synchrotron Light Source at Brookhaven National Laboratories (Upton, New York) using beamline X29 at a wavelength of 1.0 Å by participating in the mail-in program. The data were processed with the HKL2000 program (Otwinowski and Minor, 1997).

Structure determination and refinement. The structure was determined by molecular replacement using PHASER software (McCoy et al., 2007). A BLAST (Altschul et al., 1990) search of the sequences of TCR Hy.1B11 and DQ1 against the PDB database was used to find the best model for molecular replacement. Separate BLAST searches for TCR–α- and β-chains were performed and the TCR with the highest consensus score (PDB accession code 3HG1) was used for molecular replacement. DQ6 (PDB Accession Code 3HG1) was used as a starting point for screening. Crystals were obtained in multiple conditions. The final crystals for data collection were grown by the hanging-droplet vapor-diffusion method against a reservoir of 0.1 M ammonium sulfate, 8–10% PEG 8000, and 50 mM sodium citrate, pH 6.1, at 24°C. Crystals were cryoprotected by the addition of ethylene glycol to 25%. Data were collected at 100 K at the National Synchrotron Light Source at Brookhaven National Laboratories (Upton, New York) using beamline X29 at a wavelength of 1.0 Å by participating in the mail-in program. The data were processed with the HKL2000 program (Otwinowski and Minor, 1997).
affiliation accession code 1UVQ) was used as the model for DQ1 (Siebold et al., 2004). PHASER gave a clear and unambiguous solution which could be reproduced using the MOLEP program (Vagin and Teplyakov, 1997). Refinement and rebuilding were performed using crystallography and nuclear magnetic resonance system (CNS) and COOT (Emsley and Cowtan, 2004; Brünger, 2007). The overall density was good with the exception of density for residues DQ1αa 46–51, which usually form a short ascending loop in most HLA molecules that has been reported to be important for DM engagement. Stereochemical parameters of the structure were evaluated with the PROCHECK program (Laskowski et al., 1993) and found to be within reasonable limits with 91% of the residues in the most favored region and none in the disallowed regions. Buried surface area calculations were done using AREAIMOL (Lee and Richards, 1971) using a probe radius of 1.4 Å. CALCOM (Costantini et al., 2008) was used for all center of mass calculations and all figures were made with PYMOL. Atomic contacts were determined using CONTACT as implemented in CCP4i (CCP4 suite; Collaborative Computational Project, Number 4, 1994); atoms within a 4 Å distance of each other were considered to be in contact. The TCR crossing angle was calculated by drawing a vector between the center of mass of the Vα and Vβ domains and measuring the angle as it intersects a line drawn between the P1 and P9 peptide anchor residues. Relative tilt was calculated as in Teng et al. (1998).

Affinity measurements. The interaction of Hy.1B11 TCR with the DQ1–MBP85–99 complex was assessed by surface plasmon resonance using a Biacore 3000 instrument (GE Healthcare). DQ1 with a biotinylated C-terminal BirA tag was captured on a Biacore streptavidin chip. After immobilization of ~700,1,000, or 3,000 resonance units for analysis of TCR binding, solutions containing different concentrations of soluble monomeric wild-type or mutant TCR, Hy.1B11 in 10 mM Hepes, 150 mM NaCl, and 0.005% Tween 20 were injected at 15 µl/min at 25°C. Flow cells with DQ1-CLIP or DR2/MBP85–99 complexes were used as specificity controls. BLAevaluation version 4.1 was used for all data analysis. Equilibrium Kd values were obtained by nonlinear curve fitting of subtracted curves using the steady-state affinity fitting mode in BLAevaluation version 4.1. Kd values are reported as mean and standard deviation.

Analysis of peptide analogues. Proliferation assays with the Hy.1B11 T cell clone were performed using EBV transformed B cell line 9009 (DQA1*0102, DQB1*0502) as antigen-presenting cells. B cells were irradiated (5,000 rads) and treated with 50 µg/ml mitomycin C (EMD) for 30 min at 37°C. Assays were set up in 96-well U bottom plates with 5 x 10^6 T cells and 10^4 B cells in 0.2 ml of serum-free AIM-V media supplemented with 2 mM GlutaMAX. Peptides were tested in triplicates at concentrations of 0.1 and 1 µM. After 72 h of co-culture, T cell proliferation was determined by [3H] thymidine incorporation.

PDB accession no. The coordinates of the Hy.1B11–DQ1–MBP85–99 complex have been deposited under PDB accession no. 3PL6.

Online supplemental material. Fig. S1 shows electron density for critical parts of the crystal structure. Fig. S2 shows the center of mass of Vα and Vβ domains of TCR, Hy.1B11. Fig. S3 shows that the MBP85–99 peptide binds in the same register to DQ1 and DR2. Fig. S4 shows a specificity control for the TCR mutants. Fig. S5 shows alignment of microbial peptides from the National Institutes of Health (to D.K. Sethi) and the Cancer Research Institute (to D.A. Schubert). Data were collected at beamline X29 of the National Synchrotron Light Source. Financial support for the National Synchrotron Light Source comes principally from the Offices of Biological and Environmental Research and of Basic Energy Sciences of the US Department of Energy, and from the National Center for Research Resources of the National Institutes of Health.

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