γδ T Cell Receptors Recognize the Non-classical Major Histocompatibility Complex (MHC) Molecule T22 via Conserved Anchor Residues in a MHC Peptide-like Fashion*

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Background: γδ T cells recognize the non-classical MHC molecule T22 via their complementary-determining region 3δ (CDR3δ) loop.

Results: Common anchor residues in CDR3δ loops mediate binding, whereas diversity modulates T22 recognition.

Conclusion: γδ T cell receptors make use of the T22 MHC-like fold to find binding solutions for diverse CDR3δ loops.

Significance: Defining how γδ T cells recognize antigen is critical for understanding their functions in the immune response.

The molecular mechanisms by which γδ T cells recognize ligand remain a mystery. The non-classical MHC molecule T22 represents the best characterized ligand for murine γδ T cells, with a motif (W...EGYLE) present in the γδ T cell receptor complementary-determining region 3δ (CDR3δ) loop mediating γδ T cell recognition of this molecule. Produced through V(D)J recombination, this loop is quite diverse, with different numbers and chemical types of amino acids between Trp and EGYEL, which have unknown functional consequences for T22 recognition. We have investigated the biophysical and structural effects of CDR3δ loop diversity, revealing a range of affinities for T22 but a common thermodynamic pattern. Mutagenesis of these CDR3δ loops defines the key anchor residues involved in T22 recognition as W...EGYLE, similar to those found for the G8 CDR3δ loop, and demonstrates that spacer residues modulate but are not required for T22 recognition. Comparison of the location of these residues in the T22 interface reveals a striking similarity to peptide anchor residues in classically presented MHC peptides, with the key Trp residue of the CDR3δ motif completing the deficient peptide-binding groove of T22. This suggests that γδ T cell recognition of T22 utilizes the conserved ligand-presenting nature of the MHC fold.

γδ T cells are T lymphocytes characterized by expression of a somatically rearranged T cell receptor (TCR) composed of a γ and a δ chain (1). These cells are a minority population in the circulating blood and lymph, typically composing only 1–5% of circulating lymphocytes. However, γδ T cells can constitute nearly 50% of epithelial lymphocytes (2) and play a number of physiological roles, such as wound healing (3), pathogenic clearance (4–6), and tumor surveillance (5–7). The molecular mechanisms by which γδ T cells recognize cellular distress through their TCR to carry out their effector functions are largely unknown.

Both αβ and γδ TCRs are rearranged in a similar process of V(D)J gene segment rearrangement to generate a diverse pool of T cells that each express a unique TCR. For αβ T cells, this allows recognition of a similarly diverse repertoire of MHC-peptide surfaces, some of which come from antigenic peptides derived from microbial, viral, or tumorigenic sources. These peptides are presented in particular MHC molecules in part via conserved anchor residues that are shared among the MHC molecule’s peptide repertoire (8). These residues match the chemical and structural nature of the MHC molecule’s peptide-binding pockets. Recognition of the MHC and peptide composite surface is through the complementary-determining region (CDR) loops of the αβ TCR (9, 10). The CDR3 loops are particularly diverse, as they are encoded by the junctional regions of V-J (α chain) or V-D-J (β chain) recombination.

Early studies of γδ T cell recognition of MHC-like antigens demonstrated no dependence on peptide presentation, and the fewer number of V gene segments available for γδ T cell rearrangement suggested that these cells recognize ligands of a different nature (11–13). However, the ability of γδ TCRs to incorporate multiple D segments into the δ chain CDR3 loop makes the potential diversity of this loop greatest of the recombined receptors (11, 13), yet the role this sequence diversity plays in γδ T cell recognition is unclear.

Of the known ligands for γδ T cells, the murine MHC class I-like protein T22 is the best characterized (14–17). T22 is structurally similar to other class I MHCs; however, the α2 helix of T22 is incomplete, with the C-terminal end unraveled and folded back, exposing the β sheet of the platform domain (18). Unlike classical MHC molecules, T22 does not present antigen and is able to activate γδ T cells without additional cofactors.

* This work was supported, in whole or in part, by National Institutes of Health Grant R01 AI073922. This work was also supported by the Kinship Foundation Searle Scholars Award (to E. J. A.).

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2 The abbreviations used are: TCR, T cell receptor; CDR, complementary-determining region; SPR, surface plasmon resonance; ITC, isothermal titration calorimetry.
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(19). T22 is recognized by between 0.1 and 1% of γδ T cells in the mouse, including the well characterized G8 and KN6 clones (16, 19, 20). Sequence analysis of TCRs from reactive γδ T cells showed diverse use of Vδ and Vγ domains, yet the presence of a conserved motif in the CDR3δ loop composed of a tryptophan and EGYEL motif encoded within the Dδ2 segment was demonstrated to be the key component mediating T22 reactivity by single-cell sequencing (21), structural analysis (22), and later loop transfer (23). Through use of variable numbers of D segments, the length of T22 reactive γδ TCR CDR3δ loops vary, as do the number and character of the amino acids between the conserved tryptophan and EGYEL motif (21).

In this work, we sought to deconstruct how the diversity found in the naturally occurring T22 reactive γδ T cell CDR3δ sequences contributes to the recognition of T22. Specifically, we asked how variations in the CDR3δ loop in terms of total loop length, insert length, and composition modulate the interaction between T22 and the TCR. We also investigated whether the conserved motifs or the diversified residues act as the primary mediators of the interaction between the TCR and T22. The results from our study have identified a common mechanism of recognition in the sequences investigated. Finally, structural comparison of the CDR3δ loop-T22 complex with classical MHC-peptide interaction suggests that the energetically important conserved CDR3δ residues are recognized by T22 in a fashion reminiscent of how MHCs present peptide.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—T22 was expressed and purified as described previously (23). Briefly, the extracellular domains of T22 and human β2-microglobulin were coexpressed in Hi5 insect cells using the baculovirus expression system and secreted into the supernatant. Supernatants were collected, concentrated, and buffer-exchanged into 10 mM Hepes (pH 7.2), 400 mM NaCl, and 20 mM imidazole (pH 8.0). Proteins were purified using immobilized metal affinity chromatography via an engineered C-terminal hexahistidine tag. Samples were washed with 20 mM Tris (pH 8.0), 400 mM NaCl, and 20 mM imidazole (pH 8.0) and eluted with 20 mM Tris (pH 8.0), 400 mM NaCl, and 250 mM imidazole (pH 8.0). The hexahistidine tag was removed by carboxypeptidase digestion at a 1:100 mg/mg ratio, followed by a final size exclusion chromatography purification over a Superdex 200 column in Hepes-buffered saline (10 mM Hepes (pH 7.4), 150 mM NaCl, and 0.02% sodium azide). Biotinylated T22 was produced through an engineered T22 construct with a C-terminal site-specific biotinylation sequence followed by a rhinovirus 3C protease cleavage site and His6 tag for immobilized metal affinity chromatography purification as described above. The hexahistidine tag was removed by overnight digestion with rhinovirus 3C protease, desalted into 20 mM Tris (pH 8.0), and treated with BirA in the presence of biotin to covalently attach the biotin to the biotinylation sequence. The sample was purified by size exclusion chromatography as described above.

Single-chain γδ TCR fusion constructs were designed and expressed as described previously (23). In brief, the CDR3δ loop sequences of T22 reactive γδ TCRs were grafted onto the CDR3α loop of a single-chain version of the 172 αβ TCR. Both hybrids and alanine-scanning mutants were created using overlapping PCR primers containing either the loop sequence or the mutation and were sequence-verified. Hybrids were expressed by periplasmic secretion in Escherichia coli (24) and purified as described above.

Peptide Synthesis—The peptidesNH2-AWELISEGYELTT-COOH and NH2-AAELISEGAEATT-COOH were synthesized on an Applied Biosystems 433A peptide synthesizer using standard N-(9-fluorenly)methoxycarbonyl (Fmoc) chemistry. tert-Butyl-protected threonine preloaded onto Wang resin was obtained from AnaSpec. Other appropriately protected amino acids were obtained from either Midwest Biotech or AnaSpec. The peptides were cleaved from the resin with a mixture of 95% TFA, 2.5% water, and 2.5% trisopropylsiline for 1 h at 22 °C (all v/v). The resin was filtered away using a coarse glass fritted filter. The peptides were precipitated in ice-cold diethyl ether and isolated by filtration of the slurry through a medium glass fritted filter. Each peptide was then purified using a reversed phase preparative HPLC Zorbax SB-C18 column at ambient temperature, with an acetonitrile/water (both 0.1% (v/v) TFA) gradient of either 24–50% over 26 min or 10–30% over 20 min for NH2-AWELISEGYELTT-COOH and NH2-AAELISEGAEATT-COOH, respectively. The identity of AWELISEGYELTT was confirmed by MALDI-TOF-MS (calculated for C60H102N14O23 [M + H]⁺, 1511.719; found, 1512.03 (0.2 ppm)). The identity of AAELISEGAEATT was confirmed by MALDI-TOF-MS (calculated for C52H87N13O23 [M + H]⁺, 1284.604; found, 1285.63 (0.8 ppm)). The purity of both peptides was >90% as assessed by analytical HPLC under conditions similar to those noted above for preparative HPLC.

Surface Plasmon Resonance—All surface plasmon resonance (SPR) experiments were conducted using a Biacore 2000 system and streptavidin chip for ligand immobilization at 25 °C in 10 mM Hepes (pH 7.4), 150 mM NaCl, and 0.05% Tween 20. For each set of experiments, between 250 and 400 response units of biotinylated T22 were immobilized. The flow cell was then blocked with biotin. Analyte concentrations ranged between 4 and 0.03125 μM depending on the experiment. Sensograms were fitted with a 1:1 Langmuir binding model using the Biacore evaluation software. Each KD was determined by fitting both k on and k off, ΔΔG for each mutant was calculated from

ΔΔG = −RT ln(KD(mutant)/KD(wt))

where R is the gas constant, T is the temperature, and KD(mutant)/KD(wt) is the ratio of the wild-type KD to the alanine mutant KD. For peptide inhibition experiments, 100 to 0.78125 μM peptide in solution with 0.2 μM Vδ6A4b single-chain TCR was injected over the flow cell.

Isothermal Titration Calorimetry—T22 was coexpressed with β2-microglobulin using the baculovirus expression system in Hi5 insect cells, and single-chain TCRs were expressed using periplasmic secretion as described above. All proteins were purified in isothermal titration calorimetry (ITC) buffer (1X Hepes-buffered saline without azide), and peak fractions were used for ITC experiments. The protein concentrations were determined by BCA assay and A280 measurement (under non-denaturing conditions using undiluted fractions), and the average of these values was used as the protein concentration. Fractions were diluted with ITC buffer if necessary.
CDR3α Loop Sequence Variability Modulates Binding Affinity—We first used SPR to analyze the binding of each loop to T22 to assess the effect of sequence variation on binding kinetics (Fig. 2). The affinities of the loops ranged from 60 nM to 3.8 μM due to differences in both association and dissociation rates. The association rates of the loops varied across an ∼2-fold range (from $5.94 \times 10^4$ to $13.0 \times 10^4$ M$^{-1}$ s$^{-1}$), whereas the dissociation rates varied by 70-fold (from $5.1 \times 10^{-3}$ to 0.348 s$^{-1}$) (Table 1), suggesting that loop variation has a more profound effect on dissociation versus association rates. There was no direct correlation between loop or spacer length on overall affinity; $V_64_{\alpha_3}$, $V_66_{\alpha_4b}$, and $V_66_{\alpha_8}$ loop affinities were on the high end (168, 61, and 102 nM, respectively), comparable with that of G8 (∼55 nM), whereas $V_66_{\alpha_6}$ had the lowest measured affinity (3.8 μM) for T22, comparable with that of KN6 (2.7 μM). This suggests that CDR3α affinity for T22 is not a simple product of amino acid register, as both odd and even numbers of residues between Trp and EGYEL produced high-affinity binding, nor does the position of the W...EGYEL motif within the CDR3α loop (V$64_{\alpha_3}$) affect affinity substantially. Increasing the distance between Trp and EGYEL did not correlate with affinity either positively or negatively, as $V_66_{\alpha_8}$, with an eight-amino acid spacer, had one of the higher affinities for T22 (102 nM), but $V_66_{\alpha_6}$ had one of the lowest (3.8 μM).

Amino acid variation between loops of the same length had substantial effects. $V_66_{\alpha_4a}$ and $V_66_{\alpha_4b}$ differ by only one amino acid residue in the W...EGYEL spacer region (ELES versus ELIS, respectively), yet their affinities are nearly an order of magnitude different (720 and 60.6 nM, respectively). This suggests that single-amino acid differences within the spacer region can have profound effects on loop binding. This large discrepancy in affinity is predominantly due to differences in dissociation rate: $V_66_{\alpha_4a}$ has nearly a 10-fold faster dissociation rate ($0.0457$ s$^{-1}$) compared with $V_66_{\alpha_4b}$ ($0.0051$ s$^{-1}$). Thus, the sequence variability between the CDR3α loops surveyed has a clear effect on loop binding, but no clear correlation was revealed from our kinetic analysis.

CDR3α Loops Bind to T22 with Comparable Enthalpic/Entropic Contributions—Because sequence variation between the loops has a clear effect on binding kinetics (in particular, between $V_66_{\alpha_4a}$ and $V_66_{\alpha_4b}$, there is a charged (Glu) versus hydrophobic (Ile) difference), we sought to assess the enthalpic ($\Delta H$) and entropic ($\Delta S$) contributions of loop sequence diversity to T22 binding using ITC. Our ITC measurements were complicated with a consistent phenomenon whereby our stoichiometry of binding was close to 0.5, suggesting a bivalent association likely due to dimerization of T22 or our single-chain 172 TCR scaffold at the concentrations needed for ITC measurement. This translated to calculated $K_D$ values that were consistently 4–8-fold stronger than, but proportionally consistent with, the $K_D$ values calculated by SPR. Despite this issue, this analysis was informative at the level of relative comparisons between loop measurements assessing the effect of sequence and length variation on $\Delta H$ and $\Delta S$. Kinetic measurements of G8 and KN6 loop binding have been reported previously (23), so we included them in our thermodynamic analysis for comparison. The binding of all loops was enthalpically driven.
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Alanine-scanning Mutagenesis Reveals Conserved CDR3 Anchor Residues

Although we observed no direct correlation of loop or spacer length to affinity or thermodynamics of binding, sequence variation clearly plays a role in modulating these parameters. Our previous analysis of the G8 (WHISEGYEL) and KN6 (WEGYEL) loops via alanine-scanning mutagenesis (23) revealed different energetically critical residues within the W...EGYEL motif for each loop, suggesting that register of the loop amino acids may play an important role in binding. Our kinetic and thermodynamic measurements of the loop data set argue against a simple effect of amino acid register on potency of T22 recognition; therefore, other parameters appear to play a role in modulating binding strength. With our larger data set of loops, we were well positioned to dissect how sequence composition affects binding and to assess if sequence, spacing, or context plays a more important role in recognition. To this end, we performed alanine-scanning mutagenesis on four of the loops selected from our previous analysis: Vδ4_3, Vδ6A_4a, Vδ6A_4b, and Vδ6A_8. These particular loops were selected to probe the energetically important residues in three distinct contexts: 1) Vδ4_3 contains the same CDR3 sequence as the previously studied G8 loop but contains four additional amino acids before the conserved tryptophan; 2) Vδ6A_4a and Vδ6A_4b bind T22 with an ~10-fold difference in affinity but differ in amino acid composition only at two positions; and 3) Vδ6A_8 has the longest amino acid spacer segment of the selected loops (eight amino acids between Trp and EGYEL).

The effect of each alanine mutation was analyzed by SPR, and the results are reported as ΔΔG in Fig. 4A.

In all four loops investigated, mutation of the conserved tryptophan (W...EGYEL) resulted in undetectable binding (represented in Fig. 4A as red bars with +), similar to our findings with the G8 and KN6 loops. The conserved leucine (W...EGYEL) was also energetically important; in Vδ6A_4a and Vδ6A_4b bind T22 with an ~10-fold difference in affinity but differ in amino acid composition only at two positions; and 3) Vδ6A_8 has the longest amino acid spacer segment of the selected loops (eight amino acids between Trp and EGYEL). The effect of each alanine mutation was analyzed by SPR, and the results are reported as ΔΔG in Fig. 4A.

In all four loops investigated, mutation of the conserved tryptophan (W...EGYEL) resulted in undetectable binding (represented in Fig. 4A as red bars with +), similar to our findings with the G8 and KN6 loops. The conserved leucine (W...EGYEL) was also energetically important; in Vδ6A_4a and Vδ6A_4b, mutation to alanine resulted in undetectable binding, whereas in Vδ6A_8, binding was detectable but resulted in a ΔΔG of ~4 kcal, more than the contribution of the rest of the loop combined, excluding the tryptophan. The third most energetically important residue in each loop was the conserved tyrosine (W...EGYEL). In many cases, the fourth most critical residue for T22 recognition was the conserved glycine, suggesting that, in many loop contexts, flexibility was an impor-
tant factor for binding. The three critical residues, W...EGYEL, were also found to contribute most to the energetics of binding of the G8 loop (WHISEGYEL), suggesting that T22 recognition is mediated by specific anchor residues present in the loops of T22 reactive γδ TCRs containing the W...EGYEL motif.

To test whether these particular amino acid residues are required for T22 recognition, we made conservative mutations to these three residues in one of the CDR3 loops (V66A_4b) to test whether alternative residues could be tolerated at these positions. These mutant constructs were tested by SPR, and the results are shown in Fig. 4B. Both the tyrosine-to-phenylalanine and leucine-to-valine mutations were less detrimental to binding than the alanine counterparts. However, the tryptophan was still essential; no binding was observed when this position was mutated to tyrosine.

Our alanine-scanning mutagenesis strategy was effective in defining the primary loop contacts for T22 recognition; however, it also revealed the extent to which the variable residues modulate binding. In particular, alanine scanning revealed that the affinity difference between V66A_4a and V66A_4b is not due just to the Glu-to-Ile difference between the loops, but rather to the context of the sequence. Specifically, our results show that the mutation of Ile to Ala is energetically unfavorable in V66A_4b, but the Glu-to-Ala switch in V66A_4a has no penalty. Instead, we see a clear enhancement of binding when the neighboring Leu is mutated in V66A_4a but not in V66A_4b. Therefore, the local amino acid environment has a clear effect upon energetics of binding, suggesting that these positions contribute to the overall stability of binding.

Close examination of which kinetic parameters were affected upon mutation to alanine revealed an interesting pattern. For the majority of residues, mutation to alanine predominantly affected the dissociation rate (Fig. 5), consistent with our comparison across loops where dissociation rate varied the most and an indication that these mutations result in a general disruption of the stability of the complex. However, association rates were affected by alanine mutations at certain key residues, including those we have termed “anchor” residues (Tyr and Leu). Therefore, mutation to alanine at these particular positions can also affect the formation of the complex. Curiously, in the case of V66A_4a, mutation of the leucine in the spacer region to alanine (WLESEGYELA) resulted in a substantially slower dissociation rate (Fig. 5, upper left panel), suggesting that, within the context of this loop, Leu at this position favors a faster dissociation and contributes to weaker CDR3 binding.

The total length of the CDR3 loop, the length of the insert, and the amino acid type seem to have little effect on which residues are critical for binding, as our scanning revealed common anchor residues across all four loops examined despite extensive variability across these parameters. However, our results demonstrate that the diverse amino acid contexts of these loops directly modulate the overall affinity of binding.
CDR3 Anchor Residues Bind in a Peptide-like Fashion—To determine whether similarities exist between CDR3 loop binding of T22 and classical MHC-peptide presentation, we compared the positioning of the CDR3 anchor residues with that of anchor residues from classically presented peptides in MHC class I molecules. We first performed structural superposition (based on root mean square deviation minimization of Cα backbone distances) of T22 with the classical class I molecule Kb with the dEV8 peptide. Superposition was performed on the heavy chains only, with the peptide and CDR3 loop excluded from the calculation to minimize bias. The structures of T22 and Kb, with the positions of the CDR3 loop and dEV8 peptide, respectively, are shown individually in Fig. 6 (A and B). Of particular note are the equivalent positions of the key anchor residues tyrosine and leucine in the CDR3 loop and the phenylalanine and valine anchor residues in the dEV8 peptide, both buried in hydrophobic pockets above the α2 sheet platform. Fig. 6C shows the position of the dEV8 peptide relative to that of the CDR3 loop anchor residues produced via the superposition of the heavy chains. The position and orientation of these peptide anchor residues are very similar to those of the residues we have determined to be anchor residues in the CDR3 loops we evaluated, suggesting that CDR3 loop binding via the W...EGYEL motif is reminiscent of classical MHC-peptide presentation. The conserved positioning of these anchor residues is not restricted to Kb, as similar comparisons with other class I MHC-peptide complexes reveal similar anchor residue positioning (Db presentation of the NP366-N3A peptide and Ld presentation of the QL9 peptide) (Fig. 6C). Additionally, whereas the broken α2 helix of T22 leaves the groove exposed compared with a classical class I MHC, the energetically essential Trp of the CDR3 loop sits on T22 in a manner to enclose the pocket around the conserved Leu, in essence rebuilding the groove of the MHC (Fig. 6B). Thus, the W...EGYEL motif can serve as a peptide-like anchor for γδ TCR binding to T22.

Next, we sought to test whether a CDR3 peptide could bind to T22 independent of the Ig scaffold inherent in the TCR structure. A peptide consisting of the CDR3 motif of Vδ6A_4b (WT peptide), ASAWLESEGYELATYQ, and a control peptide in which the key tryptophan, tyrosine, and leucine residues were mutated to alanine, ASAAELESEGAEAATYQ, were synthesized and tested via binding competition in an SPR-based assay. When co-injected with the Vδ6A_4b CDR3 loop TCR single chain, the CDR3 loop peptide was able to inhibit binding in a
FIGURE 6. Structural comparisons between CDR3δ loop binding to T22 and MHC presenting peptide reveal conserved strategies for complex formation. A, the complex between the G8 CDR3δ loop and T22 is shown in the left panel (Protein Data Bank code 1YPZ), with T22 as a sliced surface representation showing the bisected platform and groove. The CDR3δ loop is shown in yellow, as is the contact region with T22, with side chains shown as sticks. In the right panel is the same view of a MHC class I protein (Kb) in gray presenting the deV8 peptide, shown as green sticks. Peptide contacts with Kb are also shown in green (Protein Data Bank code 2CKB). Amino acid side chain oxygens are shown in red, and nitrogens in blue. B, top view of the CDR3δ loop in T22 and the deV8 peptide in Kb. Colors are the same as described for A. The WHI residues of the G8 loop are shown as a surface representation and in pink. C, overlap of the CDR3δ anchor residues (Tyr and Leu) with the anchor residues of deV8 (left panel) and peptides NP366-N3A and QL9 presented by the class I molecules Dp and L6, respectively (Protein Data Bank codes 3FTG and 1LDP).

FIGURE 7. Peptide encoding the CDR3δ loop-binding motif effectively competes for CDR3δ loop binding. Concentration-dependent binding competition between the Vδ6A_4b loop TCR and a peptide encoding the CDR3δ loop sequence as assessed by SPR is shown in the upper panel. Sensograms correspond to a TCR concentration of 100 nM for each run, with peptide concentrations varying from 780 nM to 100 μM. A control peptide with alanines in place of the energetically important residues determined previously (Trp, Tyr, and Leu) was similarly assayed (lower panel), with no effect on TCR binding. Data are representative of one experiment. RU, response units.
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concentration-dependent manner, whereas the control peptide showed no effect, even at the highest concentration (Fig. 7). These results indicate that a peptide encoding the sequence of the CDR3δ loop is sufficient to bind to T22 and that the Ig scaffold of the TCR is not required for this binding event to occur.

DISCUSSION

Recognition of the non-classical class I molecule T22 has been the most informative system by which to study γδ T cell recognition, with functional, biophysical, and structural results providing the basis upon which to understand the recognition process. From these studies, it is clear that γδ T cells utilize a minimal portion of their TCR, the CDR3δ loop, to engage T22 and that this is predominantly mediated by the W...EGYEL motif embedded within it. This “innate-like” recognition seems simple. The presence of the motif confers reactivity; however, it is unclear how (or why) variation in these TCRs endowed through the V-D-J rearrangement process modulates reactivity to T22.

We show here that T22 reactive γδ T cells varying in their CDR3δ motif have a broad range of affinities for their ligand, as hinted at by previous tetramer staining and decay experiments (21, 25). The difference in kinetics of these interactions is mostly due to variation in the dissociation rate, varying 70-fold across the γδ TCRs examined. As dissociation rate is directly related to half-life, this suggests that the pool of γδ T cells that recognize T22 would have different thresholds for reactivity to T22. Some TCRs have high affinity for T22 (here, G8, Vδ4-3, Vδ6A_4b, and Vδ6A_8) with a corresponding slow dissociation rate, suggesting that, in vivo, the cells expressing these receptors would have a lower threshold for activation when encountering T22. Conversely, those TCRs with lower affinity and faster dissociation rates would likely have a higher threshold for activation, perhaps through increased levels of T22 on the cell surface or other unknown factors. Why the γδ T cell population would have differential reactivity to T22 is unclear; however, the inductive nature of T10, a close homolog of T22 that also can be a ligand for these γδ T cells, has been proposed previously as a reason behind this variation (20). Recruitment of the lower threshold γδ T cells may occur only under conditions that increase expression of T10 in combination with the constitutive expression of T22, leading to activation of a larger proportion of the T10/T22 reactive γδ T cell population during cellular stress or infection. Exposure to T22 during thymic development has been shown to have profound consequences on the effector fate of T22 reactive γδ T cells (25); however, it is unclear how differences in affinity for T22 may affect these parameters.

Our alanine-scanning mutational analysis of four additional CDR3δ loops has established that, in most (but not all) T22 reactive γδ TCRs, the critical anchor residues are the Trp, Tyr, and Leu residues, which are found in all T22 reactive loops. This suggests that these residues are situated much like those in the G8 CDR3δ loop, which also shares these energetically important anchor residues. In the complex structure of G8 with T22, these residues are positioned in an area much like that in peptides presented by classical MHC class I molecules, suggesting that, despite the divergence of T22 from its classical counterparts, it has maintained a presenting groove-like structure that is competent to bind a “peptide” (the EGYEL portion of the CDR3δ loop) when completed by the CDR3δ Trp and spacer residues preceding this motif. We noted that these anchor residues also appeared to tolerate some conservative changes (Tyr to Phe incurred less of a binding penalty than Tyr to Ala, and where the Leu-to-Ala mutation resulted in non-detectable binding, Leu to Val incurred only a moderate penalty), much like those seen in the variation of classically presented peptides. This model is further strengthened by our results demonstrating that a peptide encoding this motif, but not one mutated to Ala at the anchor positions, is sufficient to compete with TCR binding, suggesting that these peptides can adopt a conformation similar to that seen in our G8-T22 complex structure. Because none of the spacer residues we mutated were critical for binding but instead modulated binding, we hypothesize that variation in this region contributes to the variation in binding kinetics and thermodynamics described in this study. The one standout in our alanine-scanning analysis remains KN6, which differs from the other loops in that it lacks spacer residues between Trp and EGYEL. It is likely that KN6 is the exception rather than the rule and that structural constraints require this minimal loop to adopt a different binding conformation within the T22 groove-like structure, which also may explain the particularly low affinity of KN6 for T22 (~5–10 μM).

On the basis of the results presented here, we propose that T22 reactive γδ T cells have evolved a peptide-like recognition strategy whereby the CDR3δ loop serves as both the peptide and part of the peptide-binding groove of T22. Based on a simple motif, this strategy is similar to that of other innate-like receptors whereby certain patterns are recognized as stimulatory signals. Invariant natural killer T cells are the closest T cell analogy, whereby recognition of CD1d and its presented lipid antigens is predominantly mediated through a conserved motif in the CDR3α loop, contributed by the Jα18 segment. Variation in other loops, including that of the diverse CDR3β loop, can modulate reactivity to different lipids presented by CD1d. Here, T22 reactive γδ TCRs have variation intrinsic to the CDR3δ loop, both within and outside the W...EGYEL motif, which generates a repertoire of γδ T cells with a range of reactivity to their stimulatory ligands, T10 and T22, which may play a role in recognition of induced ligand expression, or other factors modulating γδ T cell reactivity.

Acknowledgment—We thank K. Christopher Garcia for past helpful discussions.

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