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The T cell antigen receptor α transmembrane domain coordinates triggering through regulation of bilayer immersion and CD3 subunit associations

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Summary

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Author Contributions

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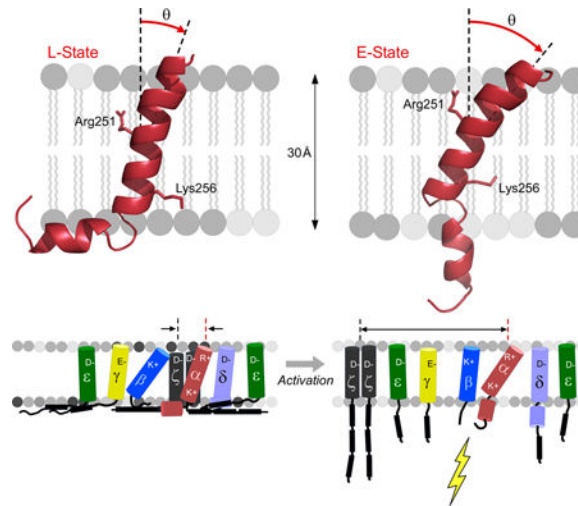
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Declaration of Interests

The authors declare no competing interests.

Initial molecular details of cellular activation following $\alpha\beta$ T cell antigen receptor (TCR) ligation by peptide-major histocompatibility complexes (pMHC) remain unexplored. We determined the nuclear magnetic resonance (NMR) structure of the TCR α subunit transmembrane (TM) segment revealing a bipartite helix whose segmentation fosters dynamic movement. Positively charged TM residues Arg251 and Lys256 project from opposite faces of the helix, with Lys256 controlling immersion depth. Their modification caused step-wise reduction in TCR associations with CD3 $\zeta\zeta$ homodimers and CD3 $\epsilon\gamma$ plus CD3 $\epsilon\delta$ heterodimers, respectively, leading to an activated transcriptome. Optical tweezers revealed that Arg251 and Lys256 mutations altered $\alpha\beta$ TCR-pMHC bond lifetimes, while mutations within interacting TCR α connecting peptide and CD36 CxxC motif juxtamembrane elements selectively attenuated signal transduction. Our findings suggest that mechanical forces applied during pMHC ligation initiate T cell activation via a dissociative mechanism, shifting disposition of those basic sidechains to rearrange TCR complex membrane topology and weaken TCR $\alpha\beta$ and CD3 associations.

Graphical abstract



In Brief

While TCR triggering is critical for adaptive immunity, how bioforces accompanying ligand binding transduce signals through the cell membrane is unknown. Brazin et al. define dynamic structural movements within the TCR α transmembrane domain linked to fundamental TCR complex mechanobiology, including subunit topological rearrangements that foster dissociation of CD3 dimers and cell activation.

Keywords

TCR; T cell activation; mechanoreceptor; NMR; EPR; optical tweezers; transmembrane domain; immunotherapy; CAR-T; transcriptome

Introduction

T lymphocytes are critical to the vertebrate adaptive immune system, performing wide-ranging immune surveillance to prevent or combat infections and cancerous transformations. The $\alpha\beta$ T cell receptor complex ($\alpha\beta$ TCR), a mechanosensor displayed on the T cell surface, mediates recognition of cellular aberrations (Das et al., 2015; Das et al., 2016; Feng et al., 2017; Kim et al., 2009; Liu et al., 2014; Mallis et al., 2015). The $\alpha\beta$ TCR is a multi-subunit protein complex composed of a disulfide-linked TCR $\alpha\beta$ heterodimer flanked by non-covalently associated dimeric CD3 subunits: CD3 $\epsilon\gamma$, CD3 $\epsilon\delta$, and CD3 $\zeta\zeta$ [reviewed in (Rudolph et al., 2006; Wang and Reinherz, 2012)]. TCR α and β each contain an extracellular variable (V) and constant (C) domain, a membrane proximal connecting peptide (CP), a single transmembrane (TM) domain, and a short cytoplasmic tail. The TCR V α V β extracellular domain module is most distal to the membrane, interacting directly with a peptide bound to a major histocompatibility complex molecule (pMHC) displayed on the surface of an antigen presenting cell (APC).

Following TCR-pMHC engagement and then force-driven conformational change and bond lifetime extension that tune antigen recognition (Das et al., 2015), there are orchestrated intracellular T cell signaling responses (Chakraborty and Weiss, 2014). The myriad of unique V modules form a repertoire of diverse T cells to interrogate the enormous immune peptidome of pathogen- and tumor-derived sequences displayed on the surface of altered cells. Each TCR specifically recognizes only one or a small number of pMHC ligands that it encounters on an APC. Since TCR α and β subunits have extremely short cytoplasmic tails lacking signaling sequences, CD3 molecules transfer cognate recognition event information into the cell (Sun et al., 2001; Sun et al., 2004). The CD3 molecules are invariant and thought to interact with the TCR α and β subunits through their extracellular and/or the TM domains to relay TCR-pMHC binding information to CD3, utilizing Immune Tyrosine Activation Motif (ITAM) regions within their cytoplasmic tails.

Formation of TCR $\alpha\beta$, CD3 $\epsilon\gamma$ and CD3 $\epsilon\delta$ heterodimers is dependent on extracellular domain interactions, while for CD3 $\zeta\zeta$, an interchain disulfide bond connects the two chains (Sun et al., 2001; Sun et al., 2004; Weissman et al., 1986). In contrast, TCR $\alpha\beta$ association with CD3 subunits requires interactions in the membrane proximal and TM domains since only weak ectodomain-mediated interactions have been detected (Birnbbaum et al., 2014; He et al., 2015; Natarajan et al., 2016). Each of the eight subunits comprising the $\alpha\beta$ TCR complex contains a single TM domain marked by the presence of highly conserved charged amino acids. A current hypothesis posits that the positions of these charged residues dictate charge-paired interactions between TCR α and β and CD3 subunits important for surface assembly, expression and signaling (Alcover et al., 1990; Blumberg et al., 1990; Call et al., 2002; Cosson et al., 1991; Manolios et al., 1990). The model suggests that basic amino acids within the TCR α and β subunits associate with acidic residues in each of the CD3 ϵ , γ , and δ TM domains to generate trimeric TCR α -CD3 $\epsilon\delta$ and TCR β -CD3 $\epsilon\gamma$ sub-complexes. A similar electrostatic charged-based coupling role is proposed for TM residues in CD3 $\zeta\zeta$ and TCR α (Call et al., 2002; Call et al., 2006). There is no direct molecular evidence for this assembly hypothesis.

To elucidate the mechanistic underpinning of these TCR TM-based associations, we focused on the two basic amino acids in the TCR α TM. We determined the structure of its TM and cytoplasmic tail (TMC) in phospholipid micelles using nuclear magnetic resonance spectroscopy (NMR). In addition, we studied the structural features in liposomes using electron paramagnetic resonance spectroscopy (EPR), permitting membrane immersion depth and intra-helical distance measurements. Lastly, we assessed the biological importance of TCR α TMC features by engineering structurally-guided mutations with each variant assembled as a component of the TCR complex on the surface of T cells. We observed that the TCR α TMC consists of a conserved sequence in vertebrates forming a bipartite helical structure whose segmentation fosters dynamic movement in the lipid environment. Mechanical force operative during pMHC ligation of the TCR is capable of toggling this conformational switch, altering TM immersion topology to impact overall $\alpha\beta$ TCR subunit associations and T cell activation.

Results

The TCR α TMC segment adopts a bipartite L-shaped helix with a flexible C-terminal region.

The 3D-structure of the TCR α TMC (residues 226–271) composed of the extracellular CP region, TM domain, and cytoplasmic tail was determined by NMR in phospholipid micelles (Figure 1A-1C) with structural restraints given in Figure S1A. The backbone RMSD was determined to be 0.38 Å for Helix 1, 0.46 Å for Helix 2, and 1.58 Å for both helices combined (Figure S1B). The N-terminal CP region (Asp226-Leu240) displays considerable flexibility and is disordered based on backbone chemical shifts. This agrees with PSIPRED prediction, and absence of the disulfide-linked connection to the TCR β subunit. In contrast to the CP region, the TM domain (Asn241-Leu268) adopts an α -helical, L-shaped structure composed of two helices interrupted by a hinge (Figure 1B, bottom). Helix 1 (Asn241-Ala258) is of sufficient length, ~26–30 Å, to transverse a cellular membrane; however, the bend (Gly259-Asn261) and subsequent reorientation of Helix 2 (Leu262-Leu268) prevents the C-terminal residues from being solvent exposed outside the lipid environment (Figure 2A and 2B). Two lines of evidence suggest the L-shaped helix is in conformational exchange with an extended helix. First, the doubling of spectral resonances observed in the ^1H - ^{15}N HSQC spectrum indicates conformational exchange between a favored bent state and an extended state (Figure 1B and 1C) at a rate slower than 5 s^{-1} , an upper bound determined by the experimental time of the ^1H - ^{15}N HSQC experiment. Second, alternate conformations of Helix 2 are observed in a subset of molecular models due to hinge flexibility. We postulate that the C-terminus becomes more solvent accessible when extended.

Sequence analysis of the TCR α segment shows that the hinge is highly conserved in mammals (Figure 1D). The CP region in mammals evolved to be ~14 amino acids long, containing a nearly 100% conserved FETDxxLN sequence, while both length and sequence conservation vary in non-mammalian vertebrates (Figure 1D and S2A). A pair of basic residues in the TCR α TMC segment, Arg251 and Lys256, are conserved in virtually all vertebrates. These same residues are conserved in an identical position in pT α , which pairs with TCR β during thymic development to form the ligand binding heterodimer of the pre-TCR (von Boehmer et al., 2003) (Figure S2B). Arg251 and Lys256 in Helix 1 are positioned

on opposite faces of the helix (Figure 1B). Helix 2 also displays high sequence conservation (Figure S2A) that continues through to the tail. The TCR α cytoplasmic tail is unusually short, predicted to comprise of only five residues. As noted below from the averaged paramagnetic relaxation enhancement (PRE) data, only two of the five residues are not membrane embedded. A survey of 507 type I integral membrane proteins (Figure 1E) on the surface of T cells in the UniProt database found that fewer than 5% have cytoplasmic tails of 10 residues or less, while fewer than 1% of 262 type I and type II cluster of differentiation (CD) proteins have cytoplasmic tails of 10 residues or less (Figure S2C and D). This short tail may facilitate a force-driven TCR α TM depth change to promote conformational change, dissociating the $\alpha\beta$ TCR complex subunits as described below.

Lys256 regulates the membrane depth of the TCR α TMC segment.

The immersion depth of TCR α TMC was investigated by NMR and EPR PRE-analyses to map the TM boundaries and geometry of the TMC segment relative to the lipid environment. Relaxation properties of solvent-exposed residues will be affected by addition of water-soluble paramagnetic ions, whereas membrane embedded residues will be largely unaffected (Figure 2A) (Respondek et al., 2007). As expected, the highly flexible extracellular CP region is exposed to solvent. Helix 1 in the TM domain and a portion of the Helix 2 appear to be micelle-embedded, but neither is deeply buried. The TCR α TMC becomes more solvent accessible at the hinge (residues 259–261) and more embedded in Helix 2, thereby reducing the solvent accessibility of the cytoplasmic tail (Figure 2A). The PRE data from the Ile, Leu and Val (ILV) methyl resonances also reveal that residue side chains in proximity to Arg251 and Lys256 are more exposed, perhaps due to the hydrophilic side chains pulling the helical TM domain towards solvent.

To corroborate observations on membrane insertion of TCR α TMC, we used EPR (Figure 2B) to measure immersion depth in DOPC:DOPG liposomes, a lipid bilayer environment distinct from detergent micelles utilized in our NMR studies. Single spin labels (R1; nitroxide spin label MTSL) were used at Val247 or Leu254 in Helix 1 or Leu266 in Helix2, within the liposome-embedded TCR α TMC to monitor responses to polar and nonpolar paramagnetic compounds thereby gauging the segment depth (Song et al., 2009). The targeted residues were immersed at relatively shallow depths in the acyl chain region (with head group defined as -5 \AA to 0 \AA and acyl chain region as $>0 \text{ \AA}$ for the liposome) (Figure 2B). The WT TCR α TMC depth is shallow in both micelle and liposome membrane systems; insertion is not a product of the experimental system but a consequence of the TM amino acid composition.

We next examined the effect of the two basic amino acids (Arg251 and Lys256) on the positioning of the TCR α TM domain in the liposome via mutation to non-polar Leu residues, generating protein segments R251L, K256L, and R251L/K256L. Mutation of Arg251 had negligible effects on liposome immersion depth as assessed by EPR (Figure 2B). In contrast, mutation of Lys256 resulted in substantial depth change for residues within the TM domain compared to the WT or R251L segments (Figure 2B). For both the K256L and R251L/K256L segments, the spin label at position 247 remained relatively shallow. However, positions 254 and 266 were considerably more immersed in the liposome. The

depth at position 254 increased substantially relative to WT for K256L and R251L/K256L. An increase in depth was also observed at position 266 relative to WT for K256L and R251L/K256L. Thus, Lys256 is essential for maintaining the shallow positioning of both helices in a membrane environment.

TCR α TM hinge mutations generate a straightened helix.

To investigate whether the TCR α TMC L-shaped configuration also contributes to the shallow depth, we engineered a straightened helix based on the NMR structure and mammalian sequence conservation in this region (Figure 1). The HSQC spectrum of the G259L/N261A mutant (Figure 2C) revealed elimination of resonance doubling; remaining chemical shifts were consistent with the minor population observed in Figure 1C. This result suggested that the minor population represented the extended conformer identified in our structural calculations. To confirm this observation, we used continuous wave (CW) EPR to measure whether the G259L/N261A mutant increased in helical length relative to the WT TCR α TMC (Figure 2D). Pairs of residues Val247 and Leu266 or Leu254 and Leu266 were R1 spin-labeled (Figure S2E). Measured distances ranged between 10–13 Å (Figure 2D) for the WT TCR α TMC segment, considerably shorter than the expected distance of approximately 20 Å for an ideal, unbroken helix. In comparison, distances measured for the G259L/N261A segment ranged between 14–19 Å, more consistent with an unbroken helix. The distance increase independently verifies our structural model of a split membrane-spanning helix for the TCR α TMC (Figure 1B). Moreover, membrane depth analysis of the straightened TM G259L/N261A mutant using single spin EPR depth measurements (Figure 2B) corroborated the distance measurements, as residues 247 and 266 were found to reside outside the liposome acyl region. The central residue 254 was slightly more immersed in the G259L/N261A liposome than in the WT TCR α liposome, indicating shallow positioning of the main body of the helix with the N-terminal end of Helix 1 and C-terminal end of Helix 2 extending from the acyl region into the headgroup for the G259L/N261A mutant. Although G259L/N261A mutation allowed the straightened segment to traverse the ~20 Å of lipid acyl chain, the L-shaped configuration alone did not drive the shallow membrane immersion depth of the TCR α TM, instead modulating TM boundaries.

Mutations within the TCR α TM affect $\alpha\beta$ TCR surface assembly and function.

The impact of helix straightening and charged residue mutations of the TCR α subunit on signaling biology was investigated through cellular-based experiments. Stable T cell lines with full-length WT or mutated TCR α (R251L, K256L, R251L/K256L and G259L/N261L) were generated. Copies of surface $\alpha\beta$ TCR were comparable between the TCR α WT and all mutant cell lines relative to the negative control (Figure 3A). Anti-TCR β clonotype and V β 5-specific mAb binding confirmed a natively folded N15TCR $\alpha\beta$ on the cell surface (Figure 3A and S3A). In contrast, the surface CD3 expression measured by staining with either anti-CD3 ϵ or anti-CD3 $\epsilon\gamma$ mAbs, was less than 2% of WT for K256L and R251L/K256L cell lines (Figure 3A & S3A) and verified by cold blocking experiments to precisely assess background staining (Figure S3B). The TCR α association with CD3 was also investigated for the mutant cell lines using the anti-TCR α antibody H28 (Figure 3A). H28 only binds to surface TCR α in the absence of CD3 $\epsilon\delta$, as its epitope is occluded by CD3 $\epsilon\delta$ and glycans attached to CD3 δ . H28 surface staining was observed for the K256L and

R251L/K256L cell lines but not for the WT or R251L cell line, confirming the presence of TCR α on the surface of the K256L cell lines lacking associated CD3 heterodimers. Hence, although the surface TCR $\alpha\beta$ heterodimers were expressed at WT copy numbers in the K256L and R251/K256L cell lines, mutation of Lys256 eliminated both CD3 $\epsilon\gamma$ and CD3 $\epsilon\delta$ from the cell surface. The flow cytometry data (Figure 3A and S3) suggest that K256 mutation dissociates TCR $\alpha\beta$ from both CD3 heterodimers. Independently derived cell lines generated for each mutant corroborated the phenotypes.

TCR $\alpha\beta$ and CD3 dimer cell surface assembly was further probed using immunofluorescence confocal microscopy (Figure 3B). Background surface staining of a negative control cell line is shown in Figure S3C. Surface expression of the TCR β subunit and the CD3 $\epsilon\gamma$ heterodimer was determined in representative cells (Figure 3B left panel). Co-localization of fluorochrome staining is readily observable on the WT cell line (Figure 3B), consistent with co-association of these molecules in the normal $\alpha\beta$ TCR complex on a T cell surface. CD3 ζ was also detected after permeabilization and staining with a CD3 ζ cytoplasmic tail antibody (Figure 3B right panel). Co-localization of CD3 ζ and TCR β at the plasma membrane demonstrates a fully assembled TCR complex, given that CD3 ζ is the last component to associate in the TCR complex (Weissman et al., 1986). Both the G259L/N261A and R251L cell lines had similar staining intensities of TCR and CD3 ζ surface molecules as WT, despite straightening of the TM segment or loss of Arg251, the latter previously thought to be required for a CD3 ζ Asp-Asp residue interaction with the TCR α Arg251 (Call et al., 2006). The TCR $\alpha\beta$ cell surface presentation and distribution on the Lys256 mutant cell lines (Figure 3B, left panel) were comparable with WT (Figure 3A). However, CD3 was completely absent on the surface of the Lys256 mutant, consistent with lack of CD3 cell surface staining by flow cytometry. CD3 $\epsilon\gamma$ intracellular staining revealed that the CD3 subunits were present as aggregates in lysosomal compartments (Figure S3D). Likewise, CD3 ζ was observed in an intracellular locale distinct from the surface plasma membrane and unassociated with TCR β (Figure 3B). Hence, the TCR α Lys256 mutation in K256L and R251L/K256L cell lines generates stable surface TCR $\alpha\beta$ expression in the absence of all co-associated CD3 dimers.

Co-association of the TCR complex subunits for WT TCR α as well as the G259L/N261A, R251L, K256L and R251/K256L mutant cell lines was examined by immunoprecipitation and immunoblot analysis. TCR α and CD3 ϵ band intensities were comparable for the WT, G259L/N261A, and R251L cell lines (Figure 3C, left two panels). However, both subunits were detected at a much lower intensity in the K256L and R251L/K256L cell lines. Despite displaying copy numbers of surface TCR $\alpha\beta$ comparable to WT, the K256L and R251L/K256L TCR $\alpha\beta$ subunits could not be readily immunoprecipitated from the cell lysates regardless of varying buffer conditions that included solubilizing additives such as SDS, positive or negatively charged detergents, or cyclodextrins, nor by using alternative TCR β antibodies for immunoprecipitation. This finding indicates that the K256L and R251L/K256L TCRs are localized in a detergent-resistant membrane fraction lacking CD3 ζ or CD3 ϵ subunits. Co-immunoprecipitation of CD3 ζ was sensitive to loss of Arg251 and to TM helical straightening (Figure 3C, right panel). As cell lysis and immunoprecipitation disrupted CD3 ζ association more readily with the R251L and G259L/N261A TCR α variants than in the WT cell line, even with the mild detergent digitonin, we conclude that

Arg251 facilitates the association of CD3 ζ to the TCR complex, as does the TM hinge. However, Arg251 and hinge residues G259 and N261 are not required for the TCR-CD3 ζ interaction *in vivo* based on the observed WT IL-2 production and immunofluorescence cell staining (Figures 3B and 4A). Disruption of residues other than Arg251 in the TCR α TMC segment, including site-specific mutation of Leu245 or Phe260, mutation of the CP region, or deletion of Helix 2 have been previously observed to reduce or eliminate CD3 ζ binding to the TCR complex (Bhatnagar et al., 2003) and references therein, collectively implying that CD3 ζ association with the TCR α TM domain is weak.

Given uniform expression of cell surface TCR $\alpha\beta$ in the absence of CD3 cell surface expression in Lys256 mutant cell lines (K256L and R251L/K256L), we posited that they would be unable to produce IL-2 upon TCR stimulation. However, we were uncertain as to whether weakening of the CD3 ζ association revealed biochemically in R251L or G259L/N261A would impact TCR functionality. IL-2 response to pMHC stimulation was monitored as a sensitive bioassay of TCR complex signaling integrity (Figure 4A). No functional impact on IL-2 production was observed with the R251L variant, nor were defects observed in either surface expression or stimulation of IL-2 production in the G259L/N261A mutant (Figure 3A and Figure 4A). In contrast, IL-2 production was essentially undetectable for the K256L and R251L/K256L T cell lines, as expected. PMA plus ionomycin stimulation of K256L and R251L/K256L T cell lines yielded robust IL-2 production, confirming response to stimulation that bypasses the $\alpha\beta$ TCR complex (Figure 4A, inset).

Gene expression differences among WT, R251L and K256L $\alpha\beta$ TCR transfectants

Since basal activation states in the WT and mutant cells might differ more broadly, we performed transcriptome analysis on a minimum of two independently derived cell lines and several subclones from WT, G259L/N261A, R251L and K256L cells using RNA-Seq. The variance between the cell lines are shown in the principal component analysis (Figure S4). Gene set enrichment analysis (GSEA) (Figure 4B) revealed that relative to WT, K256L cells were substantially enriched for signatures of oxidative phosphorylation and glycolysis that signify an increase in cellular metabolism critical for supporting energy requirements of T cell activation. Additionally, patterns representative of stimulated T cells were observed in the K256L cells relative to the WT cells. The metabolic and transcriptional activity found in the K256L cells are characteristic of late stage T cell activation, whereas the R251L cells showed features of early stage T cell signaling. For example, the R251L cells displayed an elevation in GTPase activity, known to control cytoskeletal organization and transcriptional regulation, as well as an increase in Golgi vesicle transport necessary to maintain T cell signaling during activation. The G259L/N261A cells showed little variation in gene signatures from WT cells and suggesting that an additional conformational change may be required for gene activation.

To further analyze differences between R251L and K256L cells, differential expression analysis (DESeq2) was carried out between phenotypic groups with a comparison represented in the heatmap found in Figure 4C. A subset of genes is labeled in Figure 4C and all are listed in Supplemental Table 1. Relative to K256L, R251L exhibited upregulation of genes such as *Tagln2* and *Slc7a8*, which stabilize cortical actin and participate in cellular

transport of amino acids, respectively, both critical to promote immunological synapse formation and maintain T cell activation. In addition, *Sh2d2a* and *Rgs1* were also upregulated in the R251L cell line, the former is an adaptor for Lck-signaling and the latter is involved in the regulation of GTPase activity at the cellular membrane. Other genes found to be up-regulated in R251L include those participating in TGF β signaling (*Inhba*) and in innate and adaptive T cell defense mechanisms (*Il27ra*). In contrast to R251L cells, whose transcriptome implies genes involved in early T cell activation events, the K256L mutant cells displayed differentially regulated genes corresponding to broader cellular programs. For instance, upregulated cellular metabolism genes include *Acox2*, *Atp1A2*, and *Cyp2c55* and genes upregulated and involved in transcription and/or translation include *Jun*, *Fox11* and *Nkd1*. Comparison of transcripts among mutants also revealed activation of *Lama3* necessary for cell migration and *Parp12* linked to the posttranslational modification of proteins. The transcriptome data show that R251L and K256L cell lines are in distinct states with R251L in an early stage of activation while K256L cells are in a later stage fostering broad transcription, translation and metabolic pathways linked to cell growth and proliferation. No gene signatures of immune exhaustion were evident to account for lack of K256L antigen responsiveness.

Association of TCR α with CD3 δ is governed by TCR α CP region and CD3 δ CxxC motif interaction.

Given the importance of the TCR α Lys256 residue in membrane positioning and TCR complex assembly, as well as prior publication purporting a critical charge pairing interaction of Lys256 in TCR α TM with Asp89 in CD3 δ TM domain (Call et al., 2002), the TMC of TCR α and CD3 δ was produced to study their binding interaction via NMR. Chemical shift and intensity changes of resonances occurred in the TCR α segment CP region upon addition of CD3 δ (Figure 5A and B). The combined spectral changes corroborate a TCR α -CD3 δ inter-subunit interaction involving the TCR α CP including residues in the FETDxxLN motif (aa234–241), referred to elsewhere as the TCR α CP motif (CPM) (Backström et al., 1998). Pointedly, chemical shift changes were not detected of similar magnitude in the TM segment nor in residue Lys256. Given undetected interactions between TCR α and CD3 δ TM domains, we investigated possible interactions between TCR α TMC and CD3 δ TMC membrane proximal regions. CD3 δ TMC contains the highly conserved membrane proximal CxxC motif, CQNC, which creates an intramolecular disulfide bond in each CD3 heterodimer subunit (Brazin et al., 2014). When the CD3 δ CxxC motif was mutated to SQNS (SxxS, Figure 5B) TCR α and CD3 δ interaction was diminished. Thus, the dominant mode of interaction observed between TCR α and CD3 δ segments is mediated by the TCR α CP region and the CD3 δ CxxC motif.

We also observed considerable changes in dynamics on TCR α as a result of CD3 δ binding. Upon addition of CD3 δ , the TCR α N-terminus and CP region heteronuclear ^1H - ^{15}N -NOE values (Mandel et al., 1995) became considerably more positive and hence more structured with CD3 δ binding, suggestive of convergence to a single conformational state (Figure 5C). The TM helices ^1H - ^{15}N -NOE values became more invariant throughout in the presence of CD3 δ , indicating a more uniform structure compared to the TCR α segment alone. The TCR α TMC dynamics were also monitored by measuring T_2 relaxation times (Mandel et al.,

1995). The addition of CD3 δ to TCR α TMC resulted in a considerable decrease in the T₂ time of the CP region indicative of a more structured state with negligible effects on the TM domain and the C-terminus. Combined, the T₂ relaxation data and the ¹H-¹⁵N-NOE data further support a CP interaction with CD3 δ that results in an increase in structure within the TCR α CP region (Figure 5D).

Specific binding site information was then sought on the CD3 δ segment. Spectral changes were observed in CD3 δ TMC in the presence of unlabeled TCR α TMC (Figure 5E). Several CD3 δ residues changed upon addition of TCR α TMC, with the largest changes in the membrane proximal region (His67-Ser79) that encompasses the CxxC motif (Cys71-Cys74) and in the cytoplasmic tail, located near the ITAM motif (Tyr127-Leu141; Figure 5F). Extensive chemical shift perturbations are consistent with the structural effects observed previously following disruption of the CxxC motif (Brazin et al., 2014). As with TCR α , chemical shift changes were not observed in the TM domain of CD3 δ . While we did observe chemical shift changes in L90 and T93, those residues may also be affected by conformational changes occurring at the CxxC region with TCR α binding, and not the result of a charge pairing interaction between TCR α Lys256 and CD3 δ D89. The involvement of the CD3 δ CxxC motif in the binding-site interaction is consistent with the observed disruption of the TCR α interaction when the two Cys were mutated to Ser (SxxS) (Figure 5B & S5A).

Mutations targeting the TCR α -CD3 δ interaction site result in signaling defects.

To determine the functional importance of the TCR α and CD3 δ interaction sites identified by NMR, TCR α CP mutants were generated in the full-length protein, mutating those residues having the largest spectral changes in the presence of CD3 δ (Figures 5A and S5B). The IL-2 production of WT and mutant N15TCR α mutant constructs were quantitated with the largest effect observed in the mutant cell line CPmut⁷ (Figure 5G), constructed to enhance the WT TCR α CP flexibility and target key CD3 δ binding residues without altering its length (EKSFETD to GGGSGSG). Effects of the TCR α CPmut⁷ were then compared with a CD3 δ WT and mutant cell line, CD3 δ -SxxS, in which the native CxxC motif was mutated to SxxS. The IL-2 response of the TCR α -CPmut⁷ cell line was substantially reduced (Figure 5G) with a calculated antigen EC₅₀ of 860 ng/ml versus 38 ng/ml for the WT cells. CD3 δ -SxxS also showed a reduction in IL-2 production with a calculated EC₅₀ of 193 ng/ml. Given that surface TCR expression on mutants was equivalent to WT (Figure S5C) and with comparable response to PMA plus ionomycin stimulation, reduced cytokine production is a consequence of diminished TCR-pMHC ligand triggering sensitivity.

Deleterious functional outcomes in the above mutants might be due to disrupted structural connectivity at the TCR α and CD3 δ juxtamembrane interface undermining force-dependent signal transduction. Optical tweezers (OT) were used in a single cell format (Feng et al., 2017) to characterize the mechanotransduction properties of the WT and mutant $\alpha\beta$ TCRs (Figure 6A and B). Concordant with the IL-2 functional results, OT analysis showed defects in Ca²⁺ triggering for both TCR α -CPmut⁷ and CD3 δ -SxxS transduced cell lines relative to WT (Figure 6B, top panel). The mutant cell lines were unable to trigger Ca²⁺ flux at high pMHC copy number in the absence of force, in contrast to WT TCR expressing cells where

internal actomyosin forces pulling on the TCR following pMHC ligation was sufficient. Only when substantial triggering force was applied (~21–27 pN for each mutant cell) were the mutant-transduced cell lines able to be activated by pMHC, an effect requiring a high ligand copy number (20,000 VSV8K^b per bead). Sensitivity of mutant receptor triggering was compromised compared to the WT TCR expressing cells where just 10 pN/TCR shear force and 2–29 molecules of VSV8K^b per bead readily induced activation. Thus, mutations targeting interaction sites identified by NMR produce defects in force-coupled signaling.

Antigen recognition sensitivity is enhanced by R251L but abrogated by K256L: mutations associated with TCR-pMHC bond lifetime alterations

The effects of mutation at the hinge and TM residues R251 and K256 on mechanotransduction were even more striking (Figure 6B, bottom panel). With G259L/N261A and R251L cells just 2 molecules of VSV8K^b in the OT system led to a response more rapidly than with WT cells. The duration of calcium flux tested with 2 VSV8K^b molecules was sustained for 105 sec +/- 17 sec (11 cells, +/- SD) and 63 +/- 20 sec (10 cells, +/- SD) for the G259L/N261A and R251L N15 T cells, respectively, relative to the much longer response time of 288 +/- 119 sec (8 cells, +/- SD) for WT N15 T cells. By contrast, the K256L cells were unresponsive to mechanotransduction at any pMHC density and with any pN force applied. Given loss of CD3 dimers due to K256L mutation, it is not surprising that the surface membrane TCR $\alpha\beta$ is signaling incompetent.

The OT system can also be used in single molecule, single cell (SMSC) format to measure individual TCR-pMHC bond lifetimes (Das et al., 2015). Force-bond lifetime dependence in SMSC assays was used to compare the WT and variant lines bearing TCR α mutations that alter the TCR α -CD3 δ interaction site, the interconversion of the TM helicies between the L- and E-state, and the TCR α TM membrane immersion depth and assembly (Figure 6C). The WT cell line showed similar force dependence of bond lifetimes as observed previously (Das et al., 2015; Das et al., 2016). The individual TCRs all manifest “catch” bonds, a counter-intuitive behavior in which bond strengthening occurs with application of force relative to 0 force and is manifested as longer bond lifetimes under load, with subsequent bond rupture occurring at higher forces exceeding that of the single bond strength (Das et al., 2015; Liu et al., 2014). Despite their impaired ability to facilitate T cell activation (Figure 6B), the CPmut⁷ and CD3 δ -SxxS mutants display similar force maxima as WT for catch-bond formation, ~15 pN, and maximal lifetime, ~8s. In contrast, the R251L mutant showed a slightly reduced maximal lifetime, but the catch bond force maximum shifted downward from the WT to ~10pN, suggesting that a subunit assembly defect impacts antigen recognition function. We also observed a similar effect in the reduction of the maximal force bond lifetime for the G259L/N261A mutant, further supporting the conclusion that CD3 $\zeta\zeta$ dissociation accelerates T cell activation. Most strikingly, the two K256L bearing mutants showed both a large decrease of maximal bond lifetime and attenuation of catch bond formation. Given the normal protein fold of the mutant TCRs as determined by several anti-TCR β mAbs (Figures 4A and S3A) the result suggests an inability of the force-transduction pathways to sustain necessary forces to gate ligand discrimination (Das et al., 2015). This could occur from attenuation of normal TM anchoring, modification of TM tilt geometry

upon force-based ligation and/or CD3 ζ -linked actin cytoskeletal associations necessary to transduce external force via the $\alpha\beta$ TCR complex.

Discussion

The TCR α subunit is a critical component of the $\alpha\beta$ TCR, essential for thymocyte development and mature T cell responsiveness to foreign antigens. We have determined the TCR α TM structure using solution NMR and pursued in parallel biophysical and biological approaches that link the structural details to the fundamental understanding of TCR subunit organization and activation mechanism. Our structure differs from a model of a TCR $\alpha\beta$ TM heterodimer that utilized a mutant TCR α TM segment where the vital Arg251 and Lys256 residues were mutated to Leu and disulfide crosslinking restraints were used to derive a tightly packed TCR $\alpha\beta$ TM heterodimeric structural model through computations restricting the TMs each to a single uniform helix (Krshnan et al., 2016). By contrast, we observed that the WT TCR α TM contains a mobile CP region, a membrane-spanning TM domain segmented into two helices by the hinge at Gly259-Asn261, and a short cytoplasmic tail. NMR and EPR analyses show that the TM domain exists in a dynamic bistable state exchanging between an L-shaped configuration (L-state) and a straightened configuration (E-state) facilitated by a flexible hinge. Moreover, TM residue sidechains in Helix 1, including Arg251 and Lys256, were found to have shallow immersion depths in a membrane-like environment.

The shallow position of the TCR α TM residues undoubtedly plays a regulatory role in modulating protein-protein interactions within the membrane while the L-shaped configuration of the TCR α TM contributes to its disposition. Straightening of the helical segment induced a TM residue sidechain depth change as shown for the TCR α TM G259L/N216A mutant. As a result, Lys256 and neighboring residues move deeper into the acyl chain region in the membrane. Conversion of a shallow L-shaped TM configuration to a more straightened TM that allows the Lys256 sidechain to adopt this more deeply buried membrane topology is a likely mechanism promoting TCR signaling. We propose that the straightened conformation is the primed state with force-mediated TCR-pMHC ligation inducing the switch.

Although Lys256 in the TCR α TM segment is critical in regulating the membrane depth, we found no evidence of a charge-mediated interaction between TCR α and CD3 δ . Instead, TCR α Lys256 is key in affecting the TM segment membrane disposition potentially through its positively charged sidechain amine and negatively charged lipid head groups. The mainchain likely remains embedded in the membrane while the charged sidechain is positioned in the polar lipid head group, a phenomenon is known as “snorkeling” (Strandberg and Killian, 2003). The TM domain TCR α Lys256 and, by extension, that of the TCR β subunit Lys271 appear to be positioned to facilitate a charge-based association with the lipid head groups, mitigating energetic costs of charged sidechains within the hydrophobic environment. The TCR β TM Lys271 residue may also play a critical role in maintaining $\alpha\beta$ TCR complex assembly and surface expression (Alcover et al., 1990). Although the TCR α Arg251 does not regulate TM depth, predicated on helix topology it may form an ionic interaction with the lipid headgroups and/or participate in an interaction

with CD3 $\zeta\zeta$ in the $\alpha\beta$ TCR, either via a salt link or a polar or aromatic interface (Blázquez-Moreno et al., 2017; Call et al., 2006). As described in our dissociative model below, straightening of the TCR α TM would weaken or disrupt this Arg interaction with CD3 $\zeta\zeta$, consistent with our results and with observations regarding the preTCR that readily activates early thymocytes. pT α subunit preserves the TM Arg and Lys residues but lacks a TM hinge, consistent with CD3 $\zeta\zeta$ absence in detergent lysates of preTCR complexes immunoprecipitated with anti-CD3 ϵ (Shinkai et al., 1993).

Specific juxtamembrane interactions detailed by NMR on a residue-specific basis between the CP region of TCR α and the CxxC motif of CD3 δ are consistent with the functional importance of this region shown here and in (Backström et al., 1998), establishing a mechanical link crucial to force-mediated T cell signaling without affecting TCR-pMHC bond lifetime. By contrast, the two TCR α TM charge residues control TCR-pMHC bond lifetime. Thus, the membrane proximal regions of these two subunits are involved in force relay while the TM segment residues control bond lifetime. Based on current structural and functional data, we propose a dissociative model (see graphical abstract and Figure S6) for pMHC-triggered $\alpha\beta$ TCR complex activation dependent on the distinct roles of TCR α TM Arg251 and Lys256 residues working in tandem as a part of force-driven mechanotransduction.

In the inactive state, rather loose association exists between TCR $\alpha\beta$ and CD3 TM domains, as well as between TCR α and β subunit TM domains, with the CD3 cytoplasmic tails sequestered on the plasma membrane. The TCR α subunit exists in a predominantly L-shaped configuration with Lys256 snorkeling to associate with lipid head groups. The well-conserved hinge region assists in maintaining the inactive configuration. The CD3 $\zeta\zeta$ homodimer, including the paired negatively charged Asp residues, may be in contact with the TCR α Arg251 residue (Call et al., 2006). The heterodimeric CD3 $\epsilon\delta$ and CD3 $\epsilon\gamma$ and their respective negatively charged TM residue pairs are potentially interacting with the lipid membrane positively charged choline or amino groups.

Force transduction through the membrane can initiate key changes causing the TCR α TM to alter its helical tilt, adopting a more straightened configuration, changing the position of the Arg251 sidechain and consequently disrupting an association with CD3 $\zeta\zeta$. CD3 $\zeta\zeta$ then becomes displaced from the TCR complex resulting in the first stage of activation. Concurrent with load-dependent T cell activation, interchange of membrane lipid molecules causes the cytoplasmic tails to become released from the cell membrane and available for tyrosine phosphorylation and downstream signaling (Aivazian and Stern, 2000; Deford-Watts et al., 2009; Guo et al., 2017). With the subsequent change in TCR α membrane positioning, the Lys256 side chain becomes more embedded in the acyl chains in the membrane and the CD3 heterodimers then may dissociate in this later stage of activation. Perhaps force may transiently extrude the Lys256 sidechain into the cytosol for post-translational modification by acetylation or methylation leading to greater depth in the acyl chain region post-modification. The short TCR α cytoplasmic tail will facilitate such a movement of the straightened TCR α TM helix. The clear separation of ligand binding (TCR $\alpha\beta$) and signaling (CD3) components in the $\alpha\beta$ TCR complex affords a sophistication

in receptor function that may be difficult to achieve with receptors that harbor both in one subunit, such as receptor tyrosine kinases.

Our dissociative hypothesis is consistent with observations made years ago showing that anti-clonotypic antibodies which bind to the V α V β -module recognition surface, akin to pMHC, dissociate the CD3 dimers from the TCR $\alpha\beta$ heterodimer in detergent lysates whereas anti-CD3 ϵ antibodies preserve the integrity of the entire $\alpha\beta$ TCR complex (Meuer et al., 1983). Subsequent studies have shown uncoupling of CD3 homodimers and CD3 heterodimers upon antigen exposure (Kishimoto et al., 1995; La Gruta et al., 2004). Several lines of evidence suggest that the CD3 $\zeta\zeta$ dimer can readily dissociate from the $\alpha\beta$ TCR complex. First, loss of CD3 $\zeta\zeta$ proteins from human T cells has been observed in areas of immune inflammation, particularly in associations with cancer, among other alterations, [reviewed in (Baniyash et al., 2014; Koneru et al., 2005)]. Second, weakening of CD3 $\zeta\zeta$ association described above augments the rapidity of mechanotransduction-linked calcium flux. Third, in addition to R251L, other TCR α TM mutations facilitate CD3 $\zeta\zeta$ dissociation, underscoring its weak basal interaction with the TCR subunits in the membrane environment. Fourth, early T cell transcriptional activation is a consequence of this weakening.

Chimeric antigen receptors (CARs) are recombinant proteins transduced into T cells with ligand binding activity distinct from the TCR, often employing a scFv ectodomain, a CP linker and TM segment unrelated to $\alpha\beta$ TCR components and a cytoplasmic tail consisting of an amalgam of CD3 $\zeta\zeta$ and one or more costimulatory receptor domains (Hartmann et al., 2017). In these constructs CD3 $\zeta\zeta$ is separated from TCR subunits. Nuancing of the molecular design of CARs based on evolving principles of mechanotransduction and dissociative $\alpha\beta$ TCR complex activation may extend the number of targets amenable to effective immunotherapy.

STAR Methods

Recombinant protein expression and purification

Complete methods for protein expression and purification of the ^{15}N -labeled TCR α and CD3 δ NMR samples have been described previously (Brazin et al., 2014). Briefly, N-terminal His tag, GB1 domain linked TCR α and CD3 δ TMC constructs were produced and isotopically expressed in M9 minimal media containing ^{15}N ammonium chloride as the sole nitrogen source. For expression of ILV- ^2H , $^{12}\text{C}/^{13}\text{C}$, ^{15}N TCR α samples the precursors alpha-ketoisovaleric acid (125 mg/L) and alpha-ketobutyric acid (100 mg/L) were added to the expression media 1 hour prior to IPTG induction. Expressed proteins were solubilized with SDS lysis buffer, and the CD3 δ Cys residues were oxidized and/or modified, and the protein segments were affinity purified and His-GB1 was removed by TEV digestion. The TMC segments were purified to homogeneity by size exclusion chromatography and then were TCA-precipitated and then dried. The samples were then dissolved in 30 mM Tris buffer, pH 7.0, containing 100 mM LPPG, 10% D $_2$ O, and 0.02% NaN $_3$ for NMR analysis. Deuterated LPPG (d31-LPPG obtained from fbreagents.com) was used in the solubilization buffer for the TCR α TMC sample during NMR acquisition of backbone data.

NMR spectroscopy

NMR spectra were acquired on Bruker 500, 600 and 750 or Varian 600 and 700 MHz spectrometers equipped with a 5-mm cryogenic probe. ^1H , ^{15}N heteronuclear single quantum correlation (HSQC) NMR experiments and transverse relaxation optimized spectroscopy (TROSY)-enhanced ^1H , ^{15}N HSQC spectra for the TCR α TMC, CD38 TMC, and CD38-SxxS TMC were performed at 310K. Standard three-dimensional triple resonance backbone experiments were recorded on the TCR α TMC segment for completion of the backbone resonance assignments. The sidechain assignments were completed using HCCONH and CCONH experiments and Nuclear Overhauser Enhancement (NOE) distance constraints were obtained from ILV ^{15}N - and ^{13}C - dispersed NOE experiments with a mixing time of 200 milliseconds for both experiments. Data were processed with NMRPipe and all the data were analyzed with CARA and CcpNmr software.

Residue specific PREs were determined through the acquisition of successive T_1 delay modulated ^1H - ^{15}N HSQC and ^1H - ^{13}C HSQC spectra recorded on a ILV- ^2H , ^{12}C , ^{15}N TCR α sample over the course of a titration from 0 to 10mM with Gd^{+3} ($\text{Gd}(\text{DTPA-BMA})$) purchased as Gadodiamide from Toronto Research Chemicals, Toronto, Canada. Relaxation delays of 0.15, 0.25, 0.35, 0.5, 0.75, 1, 2, 5 seconds were used in each experiment, with repeats at 0.5 and 2 seconds. Peak intensities were measured for each ^{15}N or ^{13}C -ILV resonance throughout the titration to calculate the relaxation effect (R_1) at each concentration of Gd^{+3} , and the R_1 was then used to calculate the PRE value (Franzmann et al., 2009). The calculated PRE-derived distance constraints were weighted at 30% with respect to NOEs in the structure calculation, and $\pm 3 \text{ \AA}$ was used for the upper and lower boundaries.

Chemical shift assignments and NOE assignments were completed using CARA and CcpNmr, respectively. The ARIA program was also used to determine the NOE integrals for an independent assessment of the NOE peak heights and volumes. An ensemble of folded TCR α TMC structures was generated using the following NMR determined restraints: 220 NOE restraints, 13 hydrogen bond restraints, and 68 dihedral angle restraints (obtained from the chemical shift data using TALOS). 100 structures were calculated using the simulated annealing protocol in CYANA and the 10 structures with the lowest energy target functions were chosen for deposition. PyMOL was used for structure visualization. Structural quality was assessed with PSVS and iCING.

The T_2 and heteronuclear NOE ($^{15}\text{N}[^1\text{H}]$ NOE) measurements were conducted as described (Roberts, 1993). The relaxation delays of: 16, 32, 49, 65, 81, 97, 114, ms, were used to calculate the T_2 relaxation times, with repeats on the 49 and 81 ms delays. The peak heights were integrated with CcpNmr, and T_2 times were calculated within CcpNmr. The interleaved ($^{15}\text{N}[^1\text{H}]$ NOE) experiments were recorded with a 3-s saturation delay and the $^{15}\text{N}[^1\text{H}]$ NOE values were calculated within CcpNmr.

Chemical shift mapping experiments were performed by the solubilization of ^{15}N -TCR α TMC, ^{15}N -CD38 TMC, or ^{15}N -CD38 TMC-SxxS at a concentration of 0.5 mM for TCR α and CD38-SxxS TMC, and 0.4 mM for CD38-TMC. Samples were dissolved in 100mM LPPG, 30mM Tris pH 7.0, 0.1 mM EDTA, 0.02 % NaN_3 as described (Brazin et al., 2014).

Spectra were acquired on the ^{15}N protein samples alone and then in the presence of ^{14}N protein samples at 310K using the ^1H - ^{15}N HSQC pulse sequence for ^{15}N TCR α samples and ^1H - ^{15}N TROSY-HSQC for ^{15}N CD3 β samples. Solubilized ^{14}N samples were then added to the ^{15}N samples as follows: ^{14}N - CD3 β TMC or ^{14}N - CD3 β TMC-SxxS was added in 8x excess to ^{15}N -TCR α TMC; ^{14}N -TCR α TMC was added in 7x excess to ^{15}N -CD3 β TMC; and ^{14}N -TCR α synthetic CP peptide (sequence: DATLTEKSFETDMNLFQN) was added in 8x excess to ^{15}N - CD3 β -SxxS TMC. Chemical shift and relative intensity changes were plotted vs. residue number in TCR α TMC upon addition of WT CD3 β TMC or upon addition of CD3 β -SxxS TMC and the median \pm standard deviation (SD) are 0.08 \pm 0.16 and 1.20 \pm 3.49 for the chemical shift and intensity changes, respectively. Combined chemical shift and intensity changes in ^{15}N CD3 β TMC upon addition of unlabeled TCR α TMC were plotted vs. residue number with the median \pm SD for the chemical shift and intensity changes were calculated to be 0.089 \pm 0.091 and 1.487 \pm 0.381, respectively.

TCR α multiple sequence alignment and cytoplasmic tail analysis

The multiple sequence alignment of the TCR α transmembrane region from selected vertebrate species was generated using MUSCLE from a selection of TCR α orthologues identified in the ENSEMBL and PANTHER databases. Partial reptile TCR α sequences in the alignment were obtained after BLAST searches with *Anolis carolinensis* against high throughput genome sequences at NCBI. TCR α secondary structure definition was obtained from the relevant 3D-coordinates of the NMR models using DSSP or predicted from sequence analysis using PHISPPRED.

Cytoplasmic tail residues analysis was completed on two sets of proteins obtained from the UNIPROT knowledgebase database. One set consisted of 507 single-pass type I membrane proteins expressed in T cells from mammals. The other set included 247 Human Cluster of Differentiation (CD) proteins, consisting of 218 type I and 35 type II single-pass membrane proteins. A complete list of CD proteins is available at <http://www.uniprot.org/docs/cdlist>. We used PERL scripts to retrieve the complete records of the selected proteins from UNIPROT and processed them to identify the topology and the number of cytoplasmic residues of each protein. The size distribution of the cytoplasmic tail (number of cytoplasmic residues) of the proteins was analyzed under the Jupyter Notebook environment and fitted the density distribution to cytoplasmic tail residues to a *lognormal* equation (Eq.1):

$$f(x) = \frac{1}{x\sigma\sqrt{2\pi}} \exp\left(-\left(\frac{(\text{Ln}x - \mu)^2}{2\sigma^2}\right)\right) \quad \text{Eq.1}$$

where μ is the average size of the cytosolic tails, σ is the standard deviation and π is the number PI

EPR experiments

Large unilamellar vesicles (LUVs) were prepared as described previously (Hope et al., 1985; Szoka et al., 1980). Lipids (DOPC/DOPG, 4:1 wt/wt) in chloroform were mixed in a glass

tube and dried as thin films under a stream of nitrogen gas, and were further dried using a vacuum pump for ~16 h to remove residual solvent. The lipids were resuspended in an HK buffer (20 mM HEPES, 150 mM KCl, pH 7), vortexed for 1–2 min and then subjected to 10–15 freeze-and-thaw cycles. The lipid suspension was extruded 10–15 times through a mini-extruder with a 100 nm polycarbonate membrane (Avanti Polar Lipids). Synthetic TCR α WT and mutant peptides spin labeled with MTSL (*S*-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl) methyl methanesulfonylthioate) (Toronto Research Chemicals) were obtained from the Biopolymers & Proteomics Core of Koch Institute at MIT. The peptides were dissolved in methanol (100 μ M) and subsequently co-dried along with the lipids during the liposome preparation.

The EPR measurements were carried out on a Bruker E680 EPR spectrometer (Billerica, MA) in X-band at the National High Magnetic Field Laboratory (NHMFL). Spin-spin distance data were recorded on a Bruker high-sensitivity resonator (4119HS) at 200 K. Spectra were collected at 2 mW microwave power with a field modulation frequency of 100 kHz, a modulation amplitude of one Gauss (G), and a 200 G sweep width. EPR spectra were analyzed with a Monte-Carlo/Simplex Gaussian convolution method to extract spin-spin distance (Fajer et al., 2007). For depth measurements, EPR power saturation experiments were performed on a loop-gap resonator (Molecular Specialties, Milwaukee, WI). LUV samples were loaded in gas permeable TPX capillary tubes (Molecular Specialties, Milwaukee, WI) and purged using either a stream of air or N₂ gas. EPR spectra were collected at microwave powers ranging from 0.4 to 100 mW with a modulation field of 2 G and a modulating frequency of 100 kHz. Immersion depth was calculated by the ratio of the accessibility values of O₂ to 50 mM nickel (II) ethylenediaminediacetic acid (NiEDDA). Depth standard curves were determined using lipid vesicles containing trace amounts of spin-labeled lipids (1:500 by weight) as described (Song et al., 2009).

Generation of the BW5147 T cell line

The BW5147 cell line and plasmids CD3 $\delta\gamma\epsilon\zeta$ WT pMIY and TCR $\alpha\beta$ pMIG were a gift from the Vignali lab (St. Jude Children's Research Hospital, Memphis, Tennessee). Cells were maintained in high glucose DMEM medium (Sigma-Aldrich) supplemented with 20 % (v/v) fetal bovine serum (FBS) (Sigma-Aldrich), 100 U/ml penicillin, 100 U/ml streptomycin (Life technologies), 2 mM glutamine (Life technologies). The WT mouse CD3 and N15 TCR genes in each plasmid were constructed using the viral 2A-linked system to generate multicistronic vectors for co-transfection of the CD3 and TCR genes. The N15 $\alpha\beta$ TCR is specific for the vesicular stomatitis virus nucleoprotein octapeptide (VSV8: RGYVYQGL) bound to H-2K^b (Mallis et al., 2015). The CD8 $\alpha\beta$ gene was sub-cloned into the pcDNA3.1 vector and transfected into the BW5147 cell line. The N15 TCR $\alpha\beta$ expressing cell line was generated by transfection of the CD3 $\delta\gamma\epsilon$ WT and N15 TCR $\alpha\beta$ plasmids into Phoenix-Eco packing cells (ATCC) for CD3 and TCR retrovirus production. The viral supernatants were harvested and then used to retrovirally transduce the BW5147-CD8 $\alpha\beta$ cells to incorporate the CD3 and TCR genes in that order. TCR α mutants were produced using the QuikChange II mutagenesis kit (Agilent Technologies) and plasmids were then transfected in the Phoenix-Eco packing cells for TCR retrovirus production containing a mutant TCR α gene.

Immunoprecipitation experiments

For each cell line, 2.0×10^7 cells were washed $3\times$ in TBS and lysed in lysis buffer containing: 1 % Triton X-100, 50 mM Tris pH 8.0, 150 mM NaCl, 2mM NEM, and protease inhibitor (Roche cOmplete cocktail). Cell lysis was carried-out for 30 min on ice, lysates were then centrifuged to remove insoluble material and pre-cleared with protein G beads (GE Healthcare Life Sciences, GammaBind Plus). Anti-TCR β (H57) coupled beads were then added to the lysates and incubated at 4°C for 2 hours with rotation. The beads were washed $4\times$ with cell lysis buffer and then resuspended in buffer containing 10 mM NEM, $1\times$ NUPAGE buffer, and \pm 50mM DTT. Samples were separated on 4–12% Bis-Tris NuPAGE gels (Invitrogen), transferred to PVDF membranes, and detected with the indicated antibodies. The following antibodies were used for western blotting: TCR Ca (H28), CD3 ζ (H146–968, Sigma), CD3 ϵ (gift from J.E. Coligan).

Flow cytometry

The following list of antibodies were used in FACS analysis: anti-TCR β -APC (H57–597) BD Pharmingen, anti-CD3 ϵ -APC (145–2C11) eBioscience, anti-CD3 $\epsilon\gamma$ -APC (17A2) BD Biosciences, anti-TCR $\alpha\beta$ clonotype (R53), anti-TCR V β 5 (MR9.4), Isotype Control (HTK888) BioLegend, anti-TCR Ca (H28), anti-mouse IgG-APC (poly4053) BioLegend, anti-hamster IgG-PE (polyclonal) eBioscience, anti-rat IgG-APC (poly4054) BioLegend, anti-CD3 ϵ unconjugated (2C11).

The BW5147 cells were surface stained with H57-APC and sorted for equivalent surface expressing cells on the Aria II SORP machine. APC was detected using a 660/20 band pass filter, excited with a 633nm laser and sorted with a 70 μ M nozzle. FACS analysis samples were surface stained with the indicated APC- or PE-conjugated antibodies and analyzed on the J-Fortessa machine. Dana-Farber Cancer Institute Jimmy Fund Flow Cytometry Facility.

Confocal microscopy immunofluorescence

1.0×10^5 TCR-transfected or un-transfected cells were washed twice with 3% FCS-PBS and then surfaced stained with PE-conjugated anti-TCR β (H57–597, BD Biosciences) and Alexa Fluor® 647-conjugated anti-CD3 (17A2, BD Biosciences) simultaneously for 60 min on ice. After removal of excess antibody, the PE-TCR β was amplified by rabbit anti-Phycoerythrin-R/R-PE antibody (Thermo Fisher Scientific) for 60 min on ice and then further labeled by Alexa Fluor® 594-conjugated F(ab') $_2$ fragment of goat anti-rabbit IgG (H+L) (Thermo Fisher Scientific) for 60 min on ice. Cells were then washed and filtered through 7 μ m cell strainer to remove cell aggregates before introduction via flow into a fabricated flow cell chamber. To this end, the flow cell chamber consisted of glass slide (Fisherbrand) and cover slip (FisherBrand) coated with poly-L-lysine (PLL) solution (Sigma P2890, 3% solution in ethanol). Double-sided tape was put on each edge of the glass slide to create a channel. The PLL coated cover slip was adhered to the tape face down. Cells were introduced into the channel via a pipette and kept on ice in a polystyrene tube right before microscope observation to avoid surface molecule internalization.

For CD3 ζ subunit staining, TCR β staining was carried-out first using Alexa Fluor® 594-conjugated anti-TCR β (H57, Biolegend) for 60 min on ice. Cells were then washed with 3%

FCS-PBS and permeabilized with eBioscience FoxP3 Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) according to the manufacturer's protocol. Permeabilized cells were blocked with Armenian Hamster IgG (Abcam) for 10 min on ice and then stained with FITC-conjugated anti-CD3 ζ (H146-968, Abcam) for 30 min at room temperature and then washed. The FITC-labeled antibody was then amplified with Alexa Fluor[®] 488-conjugated anti-FITC antibody (Jackson ImmunoResearch Laboratories Inc.) for 30 min on ice and then washed. In some experiments, CD3 $\epsilon\gamma$ staining was carried out simultaneously with lysosome staining using FITC-conjugated anti-mouse CD107a (LAMP-1, BD Biosciences, clone 1D4B, 1:100 dilution) plus FITC-conjugated anti-mouse CD107b (LAMP-2, BD Biosciences, clone M3/84, 1:100 dilution) overnight at 4 °C, then labeled with Alexa Fluor[®] 488-conjugated mouse anti-FITC secondary antibody for 1h at 4 °C and washed.

The Leica SP5X laser scanning confocal microscope equipped with an acousto-optical beam splitter (AOBS) system (Leica Camera AG, Wetzlar, Germany) with a 40 \times oil objective (PL APO, NA1.25) was used for image acquisition with LAS AF software. Image processing and analyses were carried out using Fiji/ImageJ (<http://rsbweb.nih.gov/ij/>).

T cell activation assays and IL-2 ELISA

The T cell activation assay was carried-out in triplicate in a 96 well plate using 2×10^5 R8 cells irradiated at 3000 rads prior to use with 2×10^5 N15 TCR $\alpha\beta$ BW cells in each well. Concentrations of the stimulatory peptide VSV8 were added to each well ranging from 50 pg/ml to 5 μ g/ml. A negative control lacked VSV8 peptide while PMA plus ionomycin was used as a positive control. The VSV8 stimulated cells and the controls were incubated for 16–18 hours overnight in a 37°C incubator, the cell supernatants were then harvested for an IL-2 ELISA assay.

The IL-2 ELISA assay was completed using the mouse IL-2 DuoSet and ancillary reagent kit 2 (R&D systems). T cell supernatants were diluted in media such that the O.D. 450 nm readings fall within the standard curve for the assay. The assay was then carried out following the kit instructions. Negative control values were subtracted from each sample point and concentrations in pg/ml were calculated from the standard curve. Measured IL-2 was plotted vs. the concentration of VSV8 peptide and fit to a 4-parameter logistic model.

RNA-Seq

A minimum of two independently derived cell lines of each mutant or WT, as well as additional subclones derived at different culture times, were used for analysis. RNA was extracted from approximately $0.25-1 \times 10^6$ cells using the QIAGEN Rneasy kit with QIAshredder treatment. Total RNA was quantified using the Qubit RNA Assay Kit (Life Tech) and RNA quality was determined on the Bioanalyzer using the RNA Pico Kit (Agilent). The NuGen Ovation Human RNA-Seq Multiplex system (NuGen, part 0341) Prep Kit, was used to target deletion of unwanted high abundance transcripts and ribosomal RNA. More than 100ng of total RNA was converted into each DNA library following the manufacturer's protocol without modification. Following library construction, DNA libraries were quantified using the Qubit High Sensitivity DNA Kit (Life Tech) and library size was determined using the Bioanalyzer High Sensitivity Chip Kit (Agilent). Finally, qPCR was

carried-out on the libraries using the Universal Library Quantification Kit for Illumina (Kapa Biosystems) and run on the 7900HT Fast qPCR machine (ABI). Libraries passing quality control were diluted to 2nM in sterile water and then sequenced on the NextSeq500 (Illumina) at a final concentration of 12pM, following all manufacturer protocols. RNA-seq data was collected at the DFCI Center for Cancer Computational Biology.

Sequencing reads were aligned to the mm10 genome using the STAR aligner and quantified as integer counts using the featureCounts summarization program of the Rsubread R package (Bioconductor). Batch correction was performed using the ComBat algorithm. DESeq2 was used to normalize the counts matrix and perform differential expression analysis between all phenotypic groups. GSEAPreranked was performed between phenotypic groups following differential expression analysis using the ranking metric k , where $k = -1 * \log_{10}(q)$ and q is the DESeq2 FDR-adjusted p-value for a gene in a given phenotypic comparison. All GSEAPreranked analyses were performed using canonical pathway gene-sets from the MSigDB database.

SMSC analysis, force-bond lifetime measurements, and induced T cell activation by optical traps

In the SMSC assay, bond lifetime measurements were carried-out using tethers constructed from half anti-biotin antibody functionalized DNA to bridge the anti-digoxigenin-coated polystyrene beads (1.0 μm in diameter, Spherotech Inc.) at one end and biotin-labeled VSV8K^b at the other end. After washing with PBST buffer (1X PBS + 0.02% (v/v) Tween-20) two times, the bead slurry was diluted 200-fold with 5 mg/mL Bovine serum albumin (BSA) (Sigma-Aldrich) in colorless DMEM medium (Sigma-Aldrich) for bond lifetime measurements. Cells were washed once with colorless DMEM medium and re-suspended to 2×10^6 cells/mL. 20 μL of the cell suspension was transferred into the flow chamber, and cells were bound to a coverslip, after 30 mins incubation at 37°C and 5% CO₂ the coverslip surface was further blocked by 5 mg/mL BSA in colorless DMEM medium. Following a 10-min incubation at 37°C and 5% CO₂, the pMHC-DNA bead slurry (~20 μL) was introduced into the same chamber. The tether-functionalized bead was trapped and brought into the cell's vicinity to form a stable tether. Detailed bead preparation procedures and methods for measuring the bond lifetime were elaborated in the previous work (Das et al., 2015).

The T cell activation SC assay was carried-out by staining the cells with Quest Rhod-4, AM (AAT Bioquest, Inc.) to observe the intracellular calcium flux during T cell triggering and then VSV8K^b coated beads with three different interfacial copy numbers (2×10^4 , 29 and 2) were used to test the triggering capacity for the different T cell lines. Procedures of VSV8K^b bead preparation and characterization, Ca²⁺ dye loading and cell triggering with optical trapped beads are available in previously published methods (Feng et al., 2017)

Supplementary Material

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Acknowledgments

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Highlights

- The TCR α transmembrane (TM) domain is a bipartite helix separated by a dynamic hinge.
- Lys256 controls the TCR α TM depth and CD3 homo- and heterodimer associations.
- The TCR α - and CD3 δ -juxtamembrane elements mediate a specific TCR α -CD3 δ interaction.
- Key residues within TCR α TM or juxtamembrane domains govern $\alpha\beta$ TCR mechanotransduction.

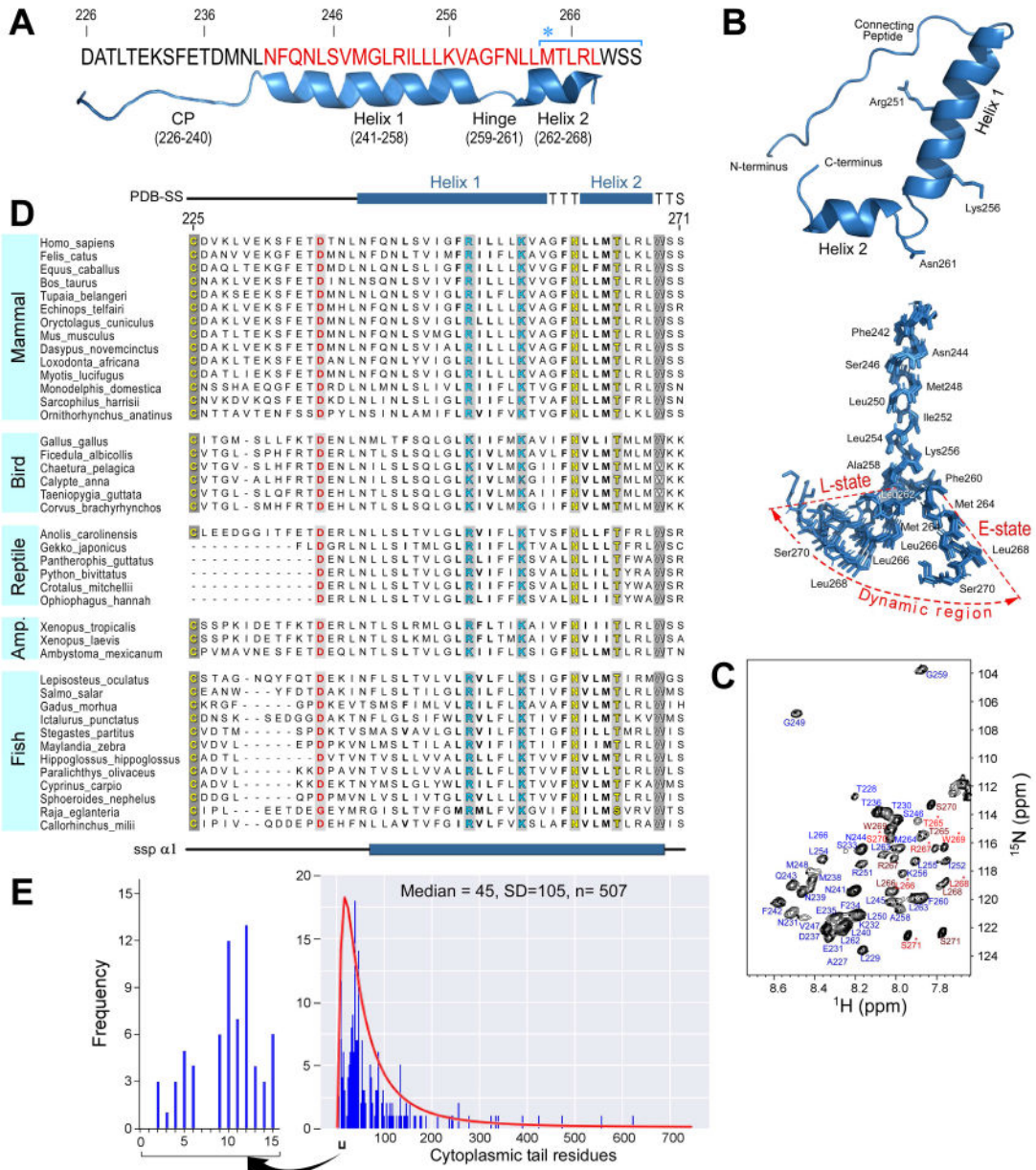


Figure 1. TCRα structure determination and sequence analysis.

A) Mouse TCRα.TMC protein sequence studied by NMR. Numbering corresponds to the full-length sequence. The structural features displayed beneath the sequence were determined experimentally: CP (226–240), Helix 1 (241–258), Hinge (259–261), and Helix 2 (262–268). The TM domain residues (241–268) are highlighted in red. Met 264 is denoted with an asterisk, and starts the sequence defined by the blue bracket to mark those residues that gave rise to peak doublings in the NMR data in (C).

B) Ribbon representation of an average conformer of the TCRα.TMC with side chains of key residues displayed. An ensemble of the 10 lowest energy conformers is shown below. The dynamic region undergoing conformational exchange is labeled and shaded to include both the bent (L)-state and extended (E)- state conformers. The flexible CP region has been

removed for clarity, and every second residue is labeled. Residues undergoing conformational exchange are labeled in each main set of conformers.

C) ^1H - ^{15}N HSQC spectrum of the TCR α TMC with complete backbone assignments. Residues exhibiting conformational exchange are labeled in two shades of red, the lighter shade designated with an asterisk represents the minor conformer.

D) TCR α TMC multiple sequence alignment from selected vertebrate species. Sequence numbering and secondary structure features depicted in the alignment correspond to the mouse TCR α TMC (residues 226–271). Conserved residues throughout all species are shown by grey shaded columns with the basic, acidic, and polar residues shown in cyan, red, and yellow font, respectively, and Trp is shown in white. A consensus secondary structure prediction (ssp α .1) is shown at the bottom of the alignment while the average secondary structure calculated from the PDB conformers (PDB-SS) is shown at the top.

E) Cytoplasmic length analysis of TM-containing T cell proteins from the UNIPROT database plotted as the frequency of occurrence versus the cytoplasmic tail length in the histogram. The black bracketed region is expanded to show those proteins with cytoplasmic tails of 15 residues or less. The log-normal distribution of the cytoplasmic tail length is plotted in red over the frequency histogram (also see Figure S1).

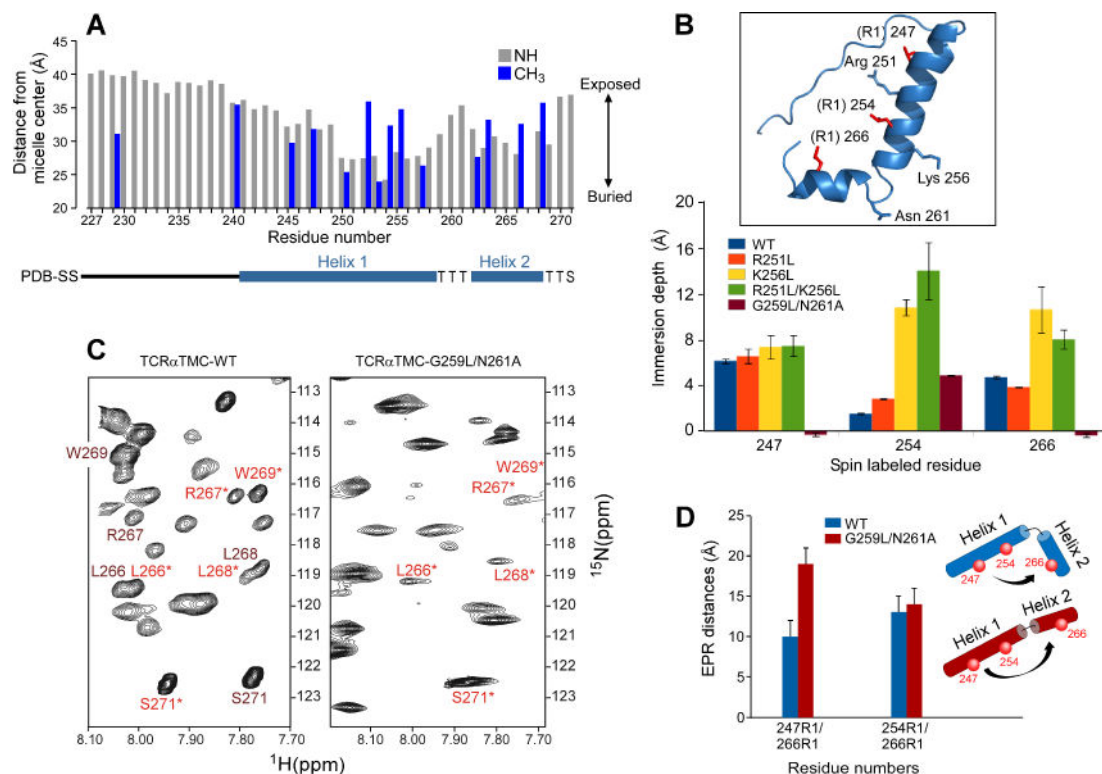


Figure 2. TCR α membrane depth analysis and generation of a straightened helix.

A) The ^{15}N backbone resonances (grey) and ^{13}C -methyl ILV resonances (blue) NMR determined membrane distance information of the TCR α TMC in LPPG micelles measured by a PRE-based analysis. A secondary structure diagram is shown below the x-axis. Residue 267 has been omitted due to spectral overlap.

B) EPR spin-labeled residues (R1) 247, 254, and 266 were used to determine the liposome immersion depths for singly labeled WT and mutant TCR α segments. Inset: Ribbon representation of the structurally determined TCR α TMC with spin-labeled residues (R1) in red and residues mutated for depth analysis highlighted in blue. Gly259 is not shown.

C) An expanded region of the ^1H - ^{15}N HSQC spectra of the TCR α TMC WT (left) and the TCR α TMC G256L/N261A mutant (right). Resonances undergoing exchange in the WT segment are labeled in dark red and in light red with an asterisk. In the G256L/N261A mutant, those remaining peaks are labeled in light red with an asterisk.

D) The EPR measured distances calculated for the TCR α TMC WT (blue) and G259L/N261A mutant (red) segments in liposomes plotted as distance versus residue numbers of the spin labeled pairs. A cartoon illustrating the position of the spin labels (red spheres) and relative orientation of Helix 1 to Helix 2 is shown for the WT segment (blue) and G259L/N261L segment (red).

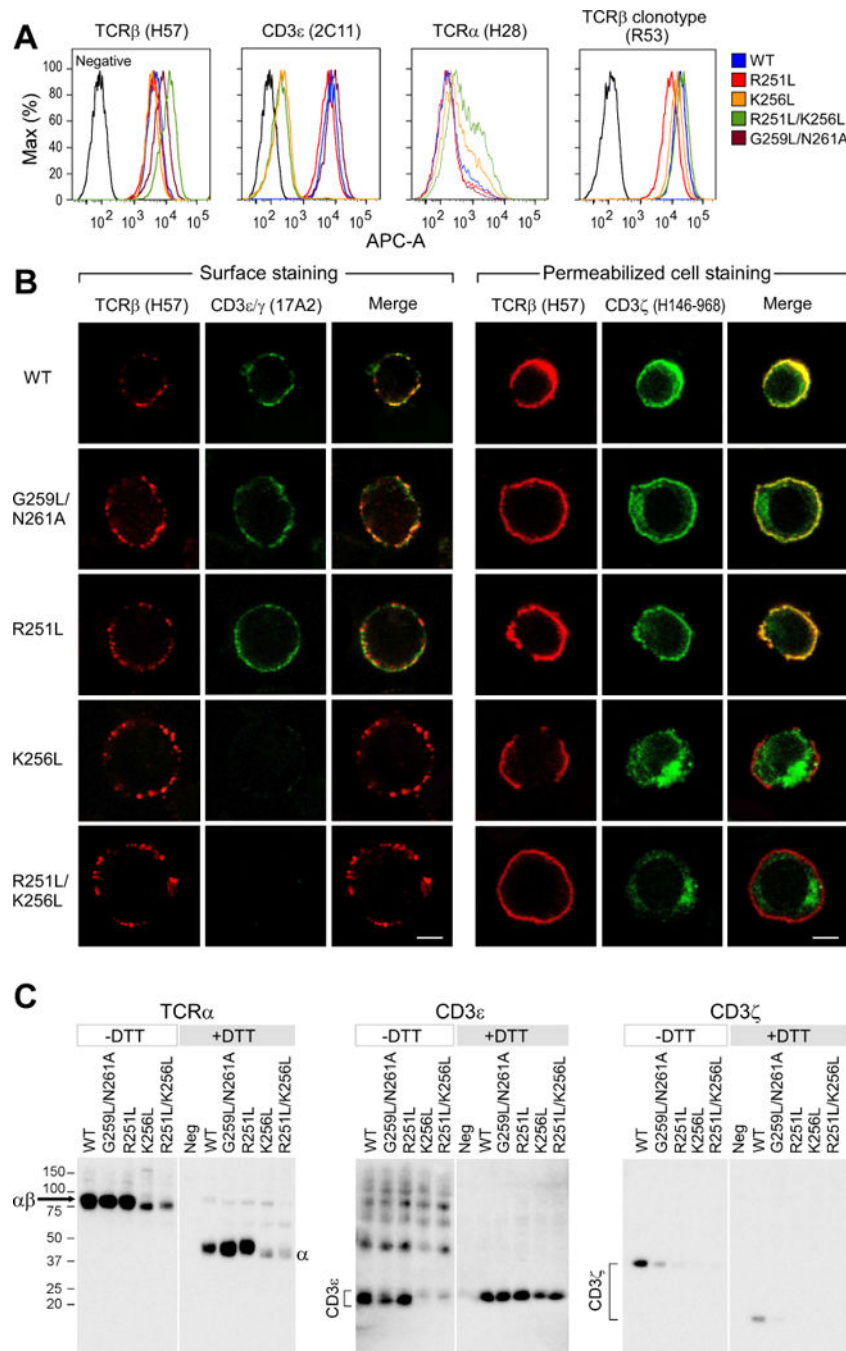


Figure 3. Biochemical analysis and surface membrane expression of TCR complex subunits in T cells transfected with wildtype versus mutant TCRα.

A) Flow cytometry analysis of the TCRβ, CD3ε, and TCRα cell surface expression using the indicated mAbs for each WT or variant TCRα chain containing cell line.

B) Confocal microscopy immunofluorescence analysis of the TCR components in the WT and mutant TCRα cell lines. A Z-plane representative slice is shown for each line with TCRβ, CD3εγ and a merge of the combined TCRβ (red) and CD3εγ (green) antibody cell surface staining results. Permeabilized cell staining results are shown for TCRβ (red) and

CD3 ζ (green) antibody intracellular staining of each cell line and a merge. Co-localization in merge appears as yellow in surface and intracellular staining. Bar = 5 μ m.

C) Immunoblot analysis of the TCR α , CD3 ϵ , and CD3 ζ co-immunoprecipitated with anti-TCR β and run under reducing (+DTT) and non-reducing (-DTT) conditions.

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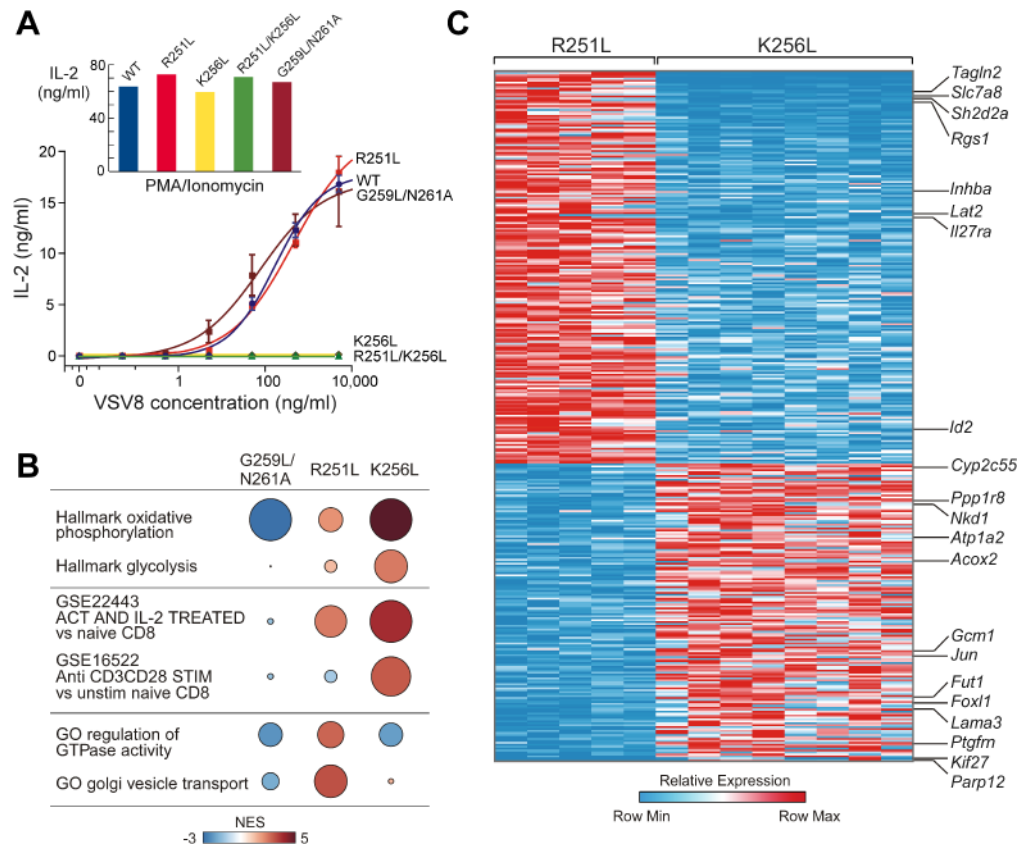


Figure 4. Activated T cell transcriptomes are differentially induced by modification of TCR α Lys256 and Arg251.

A) IL-2 ELISA results from a T cell stimulation assay using VSV8K^b for the TCR α WT and mutant TCR α cell lines. Inset: Measured IL-2 plotted vs. TCR α WT and mutant TCR α cell lines in response to PMA plus ionomycin stimulation.

B) GSEA preranked results identifying MSigDB transcriptional signatures enriched and depleted in G259L/N261A, R251L, and K256L compared to WT. The color of each is the normalized enrichment score (NES) relative to WT, and the size is negative log₁₀ false discovery rate (FDR). C) Heatmap of genes significantly differentially expressed between R251L and K256L (FDR-adjusted p-value < 0.01, log₂(fold change) > 0.5).

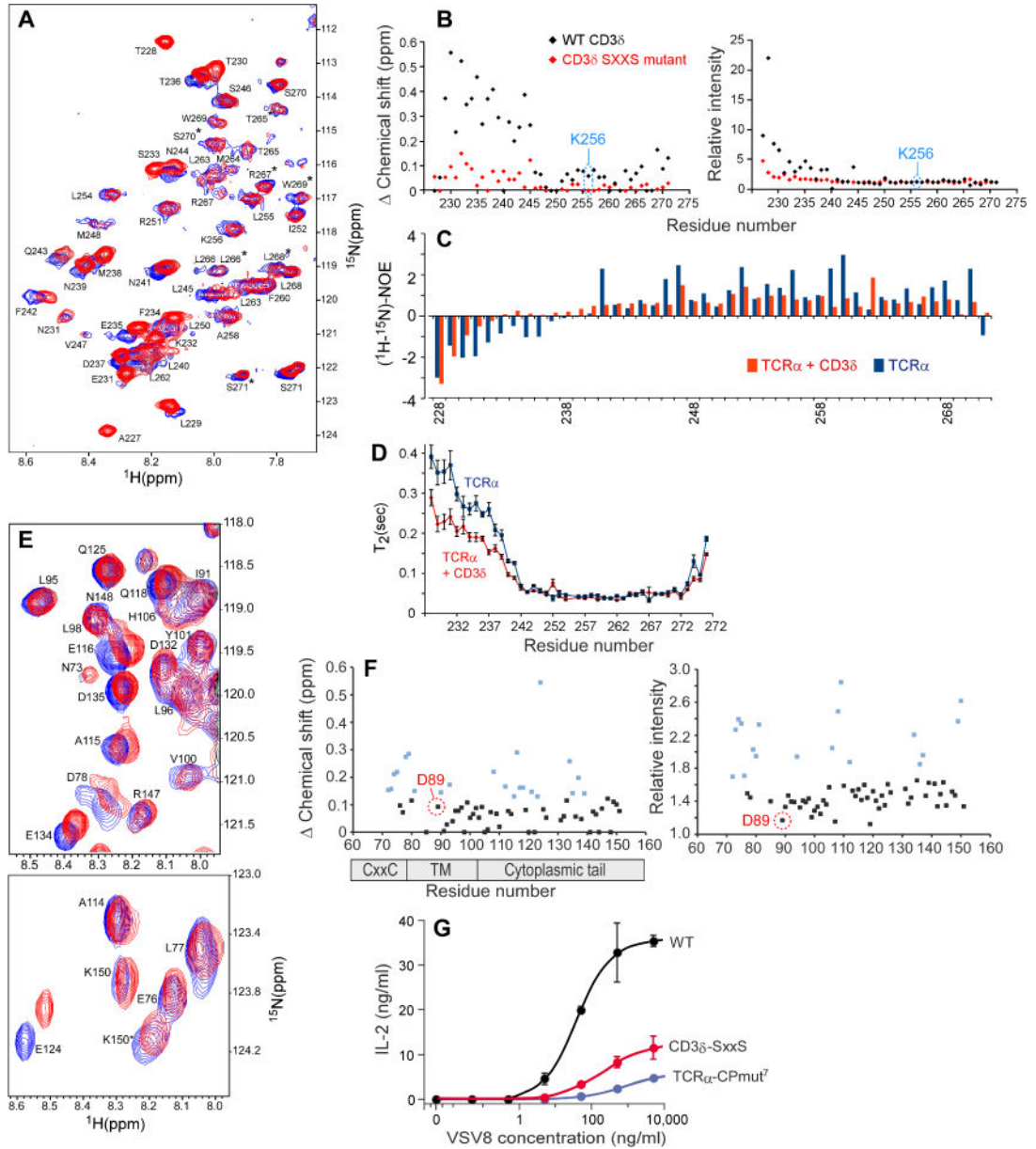


Figure 5. The juxtamembrane TCR α CP region and the CD3 δ CxxC motif mediate a key TCR α -CD3 δ intermolecular interaction.

A) ^1H - ^{15}N HSQC spectrum of ^{15}N TCR α alone (blue) or in the presence of excess unlabeled CD3 δ TMC (red). Residue specific backbone assignments are labeled and doubled resonances are denoted with an asterisk.

B) Chemical shift and intensity analysis. Left: Combined chemical shift changes plotted vs. residue number in TCR α TMC upon addition of WT CD3 δ TMC (black) or upon addition of CD3 δ -SxxS TMC (red) are shown. Right: Relative intensity changes plotted vs. residue number in TCR α TMC upon addition of WT CD3 δ TMC (black) or upon addition of CD3 δ -SxxS TMC (red) are shown.

- C) Residue specific heteronuclear NOEs were measured for the TCR α TMC segment alone (blue) or in the presence of excess CD3 δ TMC (orange) and plotted as the ^1H - ^{15}N NOE intensity versus residue number.
- D) NMR determined T_2 relaxation data of the ^{15}N TCR α TMC segment alone (blue) and in the presence of excess unlabeled CD3 δ TMC (orange). The T_2 time is plotted vs. the residue number with SD.
- E) Expanded regions of the ^1H - ^{15}N TROSY-HSQC spectrum of CD3 δ TMC alone (blue) or in the presence of 7-fold excess unlabeled TCR α TMC (red). Residue numbers correspond to the full-length mouse sequence.
- F) Chemical shift and intensity changes. Left: Combined chemical shift changes in ^{15}N CD3 δ TMC upon addition of unlabeled TCR α TMC plotted vs. residue number. Right: Relative intensity changes in ^{15}N CD3 δ TMC upon addition of unlabeled TCR α TMC plotted vs. residue number. The points highlighted in blue in each plot represent chemical shift changes and intensity changes in the top 30% with 0.13 ppm and 1.62 cut-off values for chemical shift and relative intensity, respectively.
- G) IL-2 ELISA results from a T cell stimulation assay using VSV8 peptide for the TCR α WT, TCR α -CPmut⁷ and CD3 δ -SxxS cell lines.

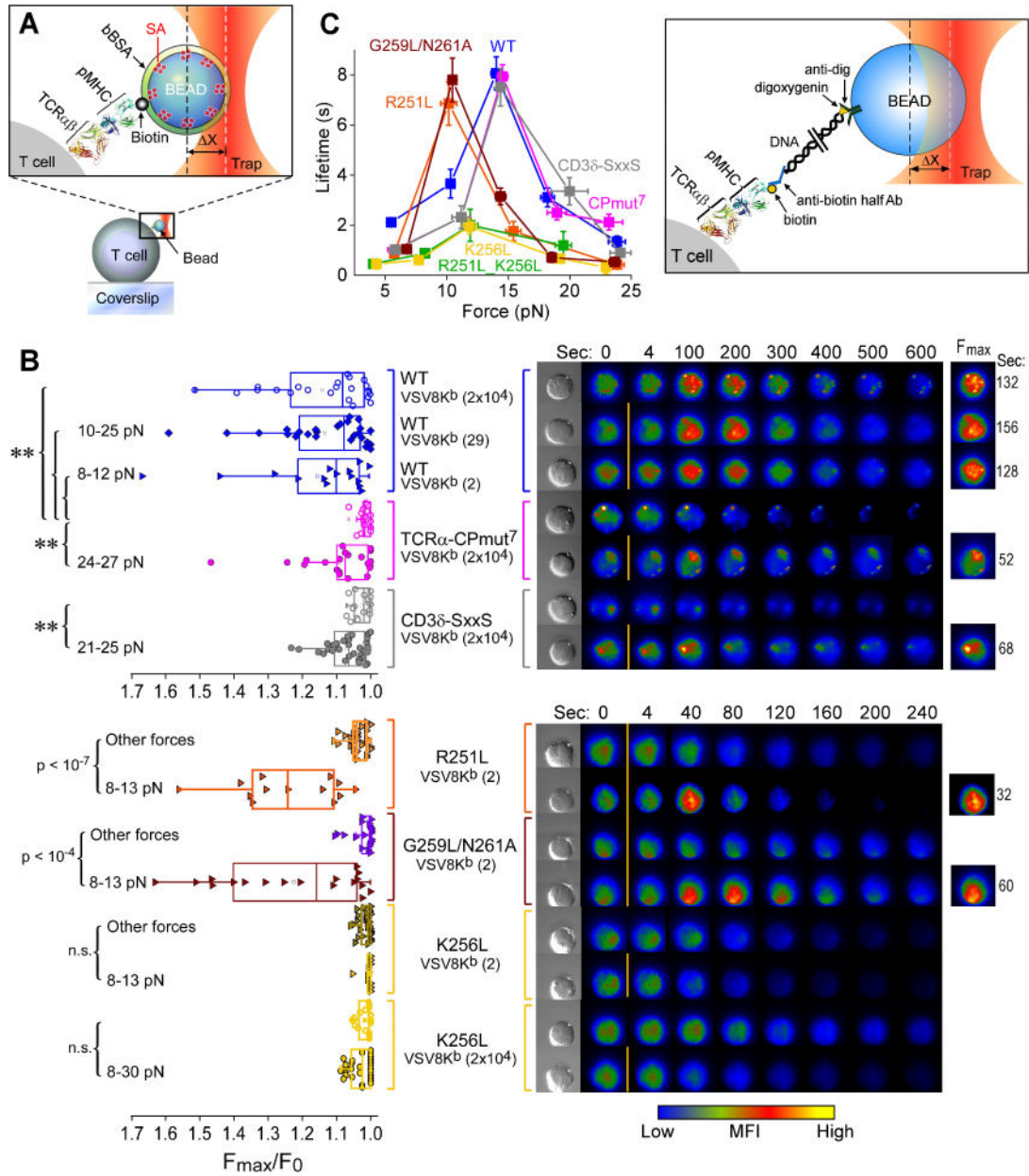


Figure 6. Functional consequences of mutations targeting the TCR α -CD36 interaction site and TCR α .TM as assessed by optical trap and fluorescence microscopy.

A) Diagram depicting the bead-cell contact in the optical trap used for applying force in the single-cell (SC) TCR triggering experiments. A streptavidin (SA)-coated polystyrene bead is bound with biotinylated pMHC, saturated with bBSA and brought into contact with an immobilized T cell. The trap force is calculated as the product of trap stiffness and displacement from the trap center (ΔX). The molecular model depicts the TCR $\alpha\beta$ heterodimer (1NFD) and pMHC (1KPU).

B) T cell activation SC analysis of the WT and mutant gene cell lines. Left: Ca^{2+} flux indicated by the ratio of maximum fluorescence intensity (F_{max}) to initial fluorescence intensity (F_0) was triggered at the indicated interfacial VSV8K^b copy numbers with applied

trapping force (5–35 pN) or without force (no label) for the different cells. Each dot in the plot represents a single cell experiment. Width of the box plot illustrates a range from 25th to 75th percentiles in score distribution. Mean and median values are shown in the square and line, respectively. The (**) represents P values < 0.01. Right: Intracellular Ca⁺² flux measured over the experiment from representative individual cells. An increase in intracellular Ca⁺² is shown by a colorization change through increases in red and yellow intensities over time. The top corresponds to the analysis of the WT, TCR α CPmut7 and CD3 δ -SxxS over 0–600 seconds vs. the bottom representing that of R251L, G259L/N261A and K256L over 0–240 seconds. The initial fluorescence signal was recorded at time zero without trapping force application (0 s) and then shear force was loaded through the VSV8K^b coated bead between 0 s and 4 s, as indicated by the orange bar. The fluorescence images as well as the times exhibiting F_{max} are shown on the right column.

C) The catch bond behavior of different TCR-expressing cell types under force is plotted as bond lifetime vs. force. Error bars represent SEM. The cartoon inset depicts SMSC tether assay for measurement of $\alpha\beta$ TCR-pMHC bond lifetimes.