

# Antigen binding to a structurally conserved TCR triggers CD3 $\zeta$ rearrangement

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# Antigen binding to a structurally conserved TCR triggers CD3 $\zeta$ rearrangement

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## Abstract

T cell receptors (TCRs) detect peptide–major histocompatibility complex (pMHC) ligands to initiate adaptive immune responses, yet how antigen recognition is coupled to cytosolic signaling remains unresolved. Here, we combine cryo-EM and single-molecule Förster resonance energy transfer (smFRET) to define the structure and dynamics of fully assembled human TCR complexes. Six cryo-EM structures reveal a conserved architecture across liganded and apo states, receptor affinity variants, and membrane environments. pMHC binding induces only local rearrangements at the binding interface but no detectable large-scale conformational change of the receptor core. In proteoliposomes containing individual receptors, cognate pMHC causes a ligand-specific shift toward increased accessibility of intrinsically disordered CD3 $\zeta$  ensembles. As a central component of early-stage T cell signaling, kinase-mediated phosphorylation produces a comparable shift and works in concert with ligand recognition to further shift the CD3 $\zeta$  population. pMHC recognition is therefore coupled to redistribution of CD3 $\zeta$  accessibility within a structurally conserved TCR complex.

**Keywords:** T cell receptor (TCR); antigen recognition; cryogenic electron microscopy (cryo-EM); single-molecule FRET (smFRET); intrinsically disordered regions (IDRs); conformational ensembles

## **Running Title**

Rigid receptor–dynamic signaling

## **In Short**

The T cell receptor complex is a structurally preorganized signaling machinery that is not activated through large-scale conformational rearrangements upon antigen binding. Instead, single-molecule measurements reveal that recognition of a single cognate pMHC shifts the conformational ensemble of cytosolic CD3 $\zeta$  signaling regions, thereby providing a mechanism by which a rigid membrane receptor transmits antigen recognition into intracellular signaling.

## **Highlights**

- pMHC binding preserves the resolved architecture of the TCR in lipid membranes
- Glycans and lipids shape the structural context of membrane-embedded TCRs
- Cognate pMHC increases accessibility of the CD3 $\zeta$  cytosolic region
- Phosphorylation by Lck shifts the CD3 $\zeta$  ensemble additively

T cells continuously survey cellular health by scanning peptide–major histocompatibility complex class I (pMHC I) molecules at the surface of nucleated cells through their eponymous T cell receptors (TCRs)<sup>1,2</sup>. The TCR complex is an obligate transmembrane heterooctamer composed of the antigen-binding module TCR $\alpha\beta$  and the signaling modules CD3 $\gamma\epsilon$ , CD3 $\delta\epsilon$ , and CD3 $\zeta\zeta$  (ref.<sup>3</sup>). While the hypervariable TCR $\alpha\beta$  heterodimer mediates pMHC recognition, intracellular signaling begins with phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) within the CD3 subunits by Src-family kinases, including the lymphocyte-specific protein tyrosine kinase (Lck)<sup>4</sup>. Phosphorylated ITAMs recruit the  $\zeta$  chain-associated protein kinase of 70 kDa (ZAP-70), initiating signaling events that ultimately reshape T cell behavior<sup>5,6</sup>.

Despite extensive biochemical and biophysical characterization of downstream signaling cascades, the molecular events that initiate TCR activation remain unresolved. One prominent model proposes that pMHC binding induces allosteric rearrangements within the TCR complex that are transmitted across the membrane through the receptor<sup>7,8</sup>. Structural and spectroscopic studies have described localized changes within TCR $\alpha\beta$ , including the C $\alpha$  AB loop, the C $\beta$  FG loop, and regions near the C $\beta$   $\alpha$ A helix and H3 region<sup>9,10</sup>. However, crystallographic comparisons of liganded and unliganded receptor fragments have generally revealed only modest differences<sup>11,12</sup>, and recent advances in cryogenic electron microscopy (cryo-EM) have validated these observations by resolving high-resolution structures of fully assembled TCR complexes<sup>13-15</sup>. Other structural models suggest that antigen recognition is transduced through altered interactions among the CD3 subunits or from force-dependent transitions generated during T cell scanning of antigen-presenting cells<sup>16-18</sup>. Several dynamic models propose that signaling can arise without major structural remodeling through spatial exclusion of large phosphatases such as CD45 from closely apposed membrane regions by kinetic segregation<sup>19,20</sup>, or formation of TCR microclusters at the immunological synapse<sup>21-23</sup>. These mechanisms do not necessarily exclude one another, but they leave open a central question: how is information at the pMHC-binding site coupled to the cytosolic signaling machinery?

High-resolution structural methods fail to capture cytosolic intrinsically disordered regions (IDRs) within the CD3 subunits, which harbor the ITAMs and are therefore essential for T cell activation<sup>24,25</sup>. The cytosolic domains of CD3 $\epsilon$  and CD3 $\zeta$  are shown to associate with the inner membrane leaflet, a property proposed to regulate the accessibility of signaling motifs<sup>26-29</sup>. A structurally conserved extracellular and transmembrane core may therefore not reflect the full conformational response of the receptor. We reasoned that pMHC recognition might be

coupled not to a large-scale structural switch in the resolved receptor core, but to redistribution of the conformational ensemble and accessibility of unresolved cytosolic signaling regions<sup>30,31</sup>. Testing this possibility requires both a membrane-embedded structural view of the receptor and a dynamic readout of the signaling domains that cryo-EM cannot resolve.

Here, we combined cryo-EM with single-molecule Förster resonance energy transfer (smFRET) to define both the structural organization and dynamic response of the intact human TCR complex in lipid membranes. We determined six cryo-EM structures of an affinity-enhanced, melanoma-associated gp100/HLA-A\*02:01-specific TCR and its parental receptor in large (~17 nm diameter) lipid nanodiscs and detergent micelles, in liganded and apo states. Across these structures, the TCR architecture remains highly conserved irrespective of ligand occupancy, receptor affinity, or membrane environment. Affinity difference arises from local optimization and partial preorganization of the pMHC-binding interface. The membrane-reconstituted structures further reveal extensive glycan shielding of the ectodomain and lipid-like densities throughout the transmembrane assembly, highlighting the structural importance of the glycocalyx and membrane environment.

We then used a reconstituted single-molecule system to interrogate the unresolved cytosolic signaling machinery. Cognate pMHC engagement produced a ligand-specific shift in the CD3 $\zeta$  conformational ensemble toward increased accessibility of its cytosolic signaling region within individual TCR complexes. Upon investigating Lck-mediated phosphorylation as a central signal transduction mechanism, we observed a similar shift in CD3 $\zeta$  as well as a complementary effect in the amplification of ligand-induced responses. Together, these findings support a framework in which a structurally conserved TCR core is coupled to a dynamic layer of cytosolic signaling domains that, in concert with extrinsic kinases, triggers intracellular phosphorylation cascades.

## Results

### pMHC binding leaves membrane-embedded TCR architecture largely unchanged

To determine how antigen recognition is transmitted through intact TCR complexes, we purified fully assembled receptors from Expi293F cells expressing TCR $\alpha\beta$  and CD3 $\gamma\delta\epsilon\zeta$ , with a C-terminal TwinStrep tag on CD3 $\zeta$  for affinity purification (Fig. 1a). Purified complexes were directly reconstituted on the affinity resin into large MSP2N2 lipid nanodiscs (~17 nm diameter)<sup>32</sup>, while glyco-diosgenin (GDN)-solubilized complexes served as detergent references (Fig. 1b; Online Methods). We analyzed the affinity-enhanced TCR<sup>GPa3b17</sup> and its parental receptor, TCR<sup>GPa<sup>b</sup>WT</sup>, which both recognize the melanoma-associated gp100 peptide (YLEPGPVTV) presented by HLA-A\*02:01 (refs.<sup>33,34</sup>). Size-exclusion chromatography (SEC) and pMHC-binding assays confirmed efficient nanodisc reconstitution and preservation of receptor function (Fig. 1c,d).

Altogether, we determined six cryo-EM structures of fully assembled TCR complexes at overall resolutions of 2.7–3.0 Å in lipid nanodiscs and 2.8–3.8 Å in detergent micelles (Fig. 1e–h; Extended Data Fig. 1a and Table 1). The ligand-bound pMHC/TCR<sup>GPa3b17</sup> assembly reconstituted in MSP2N2 nanodiscs contained all 11 polypeptide chains and adopted the characteristically tilted architecture previously observed for detergent-solubilized TCR complexes<sup>14,15</sup>. In this arrangement, the ectodomain of the TCR $\alpha\beta$  heterodimer is angled relative to the membrane plane while engaging pMHC at its apex (Fig. 1e). The corresponding apo TCR<sup>GPa3b17</sup> structure in MSP2N2 nanodiscs displayed nearly identical overall architecture (Fig. 1f).

Direct comparison of liganded and apo TCR<sup>GPa3b17</sup> revealed only minimal conformational differences upon pMHC engagement. Pairwise superposition across all TCR and CD3 subunits yielded root-mean-square deviations (RMSDs) of approximately 1 Å for most aligned residues, with differences largely confined to flexible peripheral loops and residues at the pMHC-binding interface (Extended Data Fig. 1a,b). We observed no evidence for large-scale conformational rearrangement that propagates from the TCR ectodomain into the transmembrane assembly within the steady-state conformational equilibrium of the receptor.

We next investigated whether affinity enhancement alters the overall receptor architecture. The apo structure of the parental TCR<sup>GPa<sup>b</sup>WT</sup> receptor in large nanodiscs closely resembled that of TCR<sup>GPa3b17</sup> (Fig. 1g), suggesting that the affinity-enhancing substitutions do not detectably

alter the global organization of the TCR complex. Ligand-bound and apo receptor complexes in GDN micelles likewise showed highly similar architectures (Fig. 1h). Comparisons of detergent-solubilized complexes with those reconstituted in nanodiscs revealed no detectable membrane-dependent global remodeling of the receptor (Extended Data Fig. 1a,c). Angles between transmembrane helices remained consistent in both environments, indicating a good match to the lipid bilayer thickness matched the TCR. Nanodisc-reconstituted samples also consistently yielded higher-resolution reconstructions and better-defined transmembrane maps than their detergent-solubilized counterparts (Extended Data Fig. 2–4 and Extended Data Table 1), supporting the use of a lipid bilayer for structural analysis of the intact complex.

While the parental TCR adopted an essentially indistinguishable global architecture, we investigated the origin of its substantially lower pMHC binding affinity and found that structural differences were strictly confined to the immediate pMHC-binding interface (Fig. 2a,b). Here, affinity-enhancing substitutions were concentrated predominantly within TCR $\beta$ , consistent with their greater contribution to the pMHC contacts (Fig. 2b,c). By optimizing local packing and reducing steric clashes at the binding interface, TCR<sup>GPa3b17</sup> binds with  $\sim 10^6$ -fold higher affinity to its cognate pMHC (Fig. 2d). Additionally, ligand-bound and apo TCR<sup>GPa3b17</sup> structures indicated that, in the absence of ligand, the affinity-enhanced receptor retains a partially preorganized pMHC interaction interface, which likely contributes to its high binding affinity (Extended Data Fig. 1b).

## **Glycans and lipids shape the molecular surface of the TCR**

Beyond the polypeptide architecture, cryo-EM revealed extensive non-protein densities associated with the extracellular and transmembrane regions of the TCR complex. Ten N-linked glycosylation sites were identified across TCR $\alpha\beta$ , CD3 $\gamma$ , and CD3 $\delta$ . Low-pass-filtered maps showed broad density envelopes extending across a majority of the ectodomain surface (Fig. 3a). Molecular dynamics (MD) simulations of glycan ensembles closely reproduced the experimentally observed densities, supporting far-reaching glycan shielding of the TCR ectodomains (Fig. 3a,b). Although simple glycan species were used to reflect experimental data, fully glycosylated receptors likely possess more complex glycan shells. Despite this extensive shielding covering much of the other ectodomains, the pMHC-binding apex of TCR $\alpha\beta$  remained consistently exposed across the simulated glycan ensembles. This organization preserves access to the antigen-recognition surface and may constrain other

protein interactions with the TCR ectodomains. Additional glycan-free sites were observed near the juxtamembrane regions of TCR $\alpha\beta$  and around CD3 $\epsilon$ , which lacks N-linked glycosylation sites (Fig. 3b), suggesting that the dense glycan coat evolved to shape the accessible extracellular surface of the TCR complex.

Within the transmembrane assembly, cryo-EM maps contained several lipid-like densities (Fig. 3c,d). A density at the previously described cholesterol-binding site between CD3 $\zeta'$  and CD3 $\gamma$  was observed in the outer leaflet of nanodisc-reconstituted and detergent-solubilized structures<sup>14,35</sup>. Its morphology differed between membrane environments, appearing elongated in large lipid nanodiscs and more compact in detergent. A second inner-leaflet density was larger and more phospholipid-like in nanodiscs, whereas detergent-solubilized structures contained smaller and less-defined densities. Together with additional densities embedded deeply within the transmembrane assembly throughout both leaflets (Fig. 3d), these observations indicate that the surrounding lipid environment contributes directly to the structural organization of the TCR, with glycans shaping the accessible extracellular surface and lipids occupying defined sites within the transmembrane architecture. The identities and functional contributions of individual densities remain to be established.

To assess the effect of membrane confinement on the TCR structure, we reconstituted complexes into smaller MSP1D1 nanodiscs (~10 nm diameter), which provide a more restricted lipid environment than large MSP2N2 nanodiscs (~17 nm diameter; Extended Data Fig. 5a–f). Although reconstitution efficiency was comparable, MSP1D1-reconstituted samples yielded reconstructions with lower resolution (~4 Å) and pronounced orientation bias. The TCR complex can therefore be accommodated in smaller nanodiscs, but the restricted membrane environment limits structural quality and conformational sampling. Together with the extensive lipid densities intercalated throughout the transmembrane assembly, these findings demonstrate that the surrounding membrane is not merely a passive environment but an important structural determinant of the TCR complex assembly.

### **Cognate pMHC binding triggers CD3 $\zeta$ rearrangement at the cytosolic face of the TCR**

The cryo-EM reconstructions define the architecture of the membrane-embedded receptor in liganded and unliganded states, but do not resolve its intrinsically disordered cytosolic signaling regions, most notably the CD3 $\zeta$  homodimer. Previous studies suggest that cytosolic regions of CD3 $\epsilon$  and CD3 $\zeta$  associate with the inner leaflet of the plasma membrane in resting

TCRs, thereby regulating ITAM accessibility<sup>26-29</sup>. We therefore sought to determine whether pMHC recognition at the extracellular face of the receptor is accompanied by change in the conformational ensemble of these as yet unresolved cytosolic regions by altering their membrane association, accessibility, or dynamics.

To address this question, we adapted the recently established smFRET sensor OppA to monitor CD3 $\zeta$  accessibility in TCR-containing proteoliposomes (Fig. 4)<sup>36,37</sup>. We engineered a C-terminal binding motif (RDMPIQAF) in full-length CD3 $\zeta$  of TCR<sup>GPa3b17</sup> (TCR<sup>QAF</sup>) and co-reconstituted the receptor complex into liposomes with fluorescently labeled OppA (Fig. 4a and Extended Data Fig. 6). Binding to the engineered CD3 $\zeta$  motif causes OppA to undergo a conformational transition that is reported by an upshift in FRET efficiency between donor (AF555) and acceptor (AF647) fluorophores. The assay therefore measures accessibility of the engineered CD3 $\zeta$  terminus within its membrane-associated conformational ensemble.

Single-molecule measurements of CD3 $\zeta$  dynamics were acquired by total internal reflection fluorescence microscopy with alternating-laser excitation (TIRF-ALEX; Fig. 4b,c). Proteoliposomes were immobilized through biotinylated lipids, leaving receptors freely diffusible within the lipid bilayer to avoid direct mechanical constraints or pulling on the TCRs. Different amounts of receptors per liposome were reconstituted, and incorporation of individual TCR complexes was verified from photobleaching steps of singly fluorophore-labeled pMHC (Figures 4d and Extended Data Fig. 7). Here, one-step photobleaching indicated presence of individual TCRs in 92.7% of proteoliposomes.

In the absence of TCR complexes, OppA remained predominantly in a low-FRET state and was unaffected by pMHC addition, showing that the external ligand did not alter sensor behavior (Fig. 4e). Similarly, proteoliposomes containing individual unliganded TCR complexes maintained OppA predominantly in a low-FRET state, consistent with limited accessibility of the engineered CD3 $\zeta$  terminus (Fig. 4f). Addition of cognate pMHC to single TCR-containing proteoliposomes induced a pronounced shift of the OppA sensor toward the high-FRET state, revealing a ligand-specific increase in CD3 $\zeta$  accessibility (Fig. 4f). This transition was comparable in proteoliposomes containing individual or multiple TCR complexes, indicating that antigen-induced rearrangement of the CD3 $\zeta$  conformational ensemble requires only a single TCR and does not depend on receptor clustering (Fig. 4g). In contrast, non-cognate pMHC<sup>NY-ESO-1</sup> failed to induce a FRET shift while differing only in the sequence of its presented peptide (Fig. 4h). Cognate pMHC recognition by TCR $\alpha\beta$  is therefore

functionally coupled to a ligand-specific increase in CD3 $\zeta$  accessibility at the single-receptor level.

### **Lck equally shifts CD3 $\zeta$ accessibility and enhances pMHC-induced response**

Tyrosine phosphorylation of CD3 $\zeta$  ITAMs by Src-family kinases, predominantly Lck, is an essential early event in TCR signaling that governs the fate of opposing antigen presenting cells<sup>38</sup>. Recognition of cognate pMHC molecules classically leads to co-receptor recruitment and delivery of associated Lck. Alternatively, cytosolic Lck is recruited directly to emerging immunological synapses to initiate ATP-dependent phosphorylation of ITAMs. Phosphorylated ITAMs then recruit downstream effectors such as ZAP-70 to propagate the activation signal. Recent studies have shown that phosphorylation by Lck weakens the electrostatic association of CD3 $\zeta$  with negatively charged membrane lipids<sup>28</sup>, theoretically rendering it more accessible for binding of cytosolic proteins. However, the correlation between phosphorylation and accessibility of CD3 $\zeta$  during this process remains ambiguous.

Given the crucial role of Lck as the primary activating kinase in TCR signaling, we adapted our smFRET assay to investigate its impact on CD3 $\zeta$  conformational dynamics. By co-encapsulating Lck in proteoliposomes, we aimed to reconstitute the early T cell activation machinery in a minimal format. We utilized the self-inserting nanopore  $\alpha$ -hemolysin to permit diffusion of ATP across the membrane<sup>39</sup>, and included the smFRET sensor OppA to track CD3 $\zeta$  dynamics. To dissect the individual contributions of Lck-mediated phosphorylation and cognate pMHC recognition, experiments were performed with liposomes containing sub-stoichiometric (low) or stoichiometric (high) amounts of Lck before and after addition of pMHC.

In the absence of TCR complexes, ATP or cognate pMHC did not alter the low-FRET OppA population in Lck-containing proteoliposomes, excluding an artifactual response to  $\alpha$ -hemolysin, ATP, or ligand (Fig. 5a). In individual TCR-containing liposomes at low Lck occupancy, ATP addition produced a partial shift toward the high-FRET population, consistent with increased CD3 $\zeta$  accessibility under phosphorylating conditions (Fig. 5b). Subsequent cognate pMHC binding further increased the high-FRET population as previously seen for Lck-free liposomes (see Fig. 4f). At stoichiometric Lck occupancy, addition of ATP resulted in a pronounced high-FRET population that approached the ligand-induced FRET shift in the absence of Lck (Fig. 5c). Subsequent binding of cognate pMHC led to further increase of the

high-FRET population, marking the largest shift in CD3 $\zeta$  dynamics and indicating an additive effect between both signals.

Collectively, these experiments show that Lck-mediated phosphorylation and cognate pMHC recognition independently modulated the CD3 $\zeta$  conformational ensemble. Both stimuli caused a comparable increase in the accessibility of CD3 $\zeta$ , and combination of both signals induced a synergistic effect to produce populations shifted further than before. These findings place phosphorylation and ligand recognition at different signaling layers that work in concert to facilitate TCR triggering.

## Discussion

A central, unresolved question in T cell biology is how extracellular pMHC recognition is transmitted across the plasma membrane to initiate signaling<sup>5,38</sup>. By combining high-resolution cryo-EM with single-molecule analyses of cytosolic dynamics, we present evidence that cognate pMHC recognition is associated with a ligand-specific redistribution of CD3 $\zeta$  accessibility while the static TCR architecture remains largely conserved across structural states. These observations support a model that unifies previously conflicting theories by showing that structural rigidity of the TCR complex and dynamic regulation of signaling are not mutually exclusive but rather represent complementary features of the same activation mechanism<sup>40-42</sup>.

Our six structures show that liganded and apo receptors, parental and affinity-enhanced variants, and nanodisc- and detergent-reconstituted complexes share a highly similar architecture. pMHC binding produces local changes at the interaction interface, but no detectable large-scale rearrangement through the ectodomain or transmembrane assembly. This conclusion does not exclude transient, rare, or force-dependent motions that may fall outside the resolution and conformation sampling of the present reconstructions. Rather, it argues against a model in which antigen recognition must be propagated through a stable, large-scale structural switch in the resolved receptor core, and instead proposes that the TCR exists in a preorganized conformational state posed for antigen recognition and signaling initiation<sup>14,15,43</sup>.

The affinity-enhanced receptor illustrates how ligand-binding energetics can be altered without disturbing this conserved architecture. It achieves significantly improved pMHC binding affinity through localized optimization of the antigen-binding surface rather than through

global conformational change. Affinity-enhancing substitutions minimize steric clashes, improve shape complementarity, and stabilize a partially preorganized binding interface even in the absence of ligand. While high-affinity TCRs may raise concerns regarding cross-reactivity, the affinity-enhanced receptor demonstrated strict specificity for its cognate pMHC ligand in our single-molecule experiments. These observations suggest that highly specific affinity enhancement in therapeutic receptor engineering can be achieved through targeted remodeling of local pMHC-contacting residues<sup>34,44</sup>.

Beyond protein architecture, our membrane-reconstituted structures further emphasize that the TCR complex is not solely a multi-protein assembly, and present glycans and membrane lipids as important structural features of the intact TCR. We show how the accessible molecular antigen-scanning surface of the TCR complex is constrained by surrounding glycans, which have been shown to impact pMHC recognition behavior<sup>45</sup>. The surrounding membrane contributes to stabilizing and organizing the assembly, with lipid-like densities present in the core of the transmembrane helix bundle. In contrast to previous studies, we did not observe a clear preference for cholesterol binding to the TCR transmembrane region in any of the structures<sup>35,46,47</sup>. In structures of detergent-solubilized complexes, densities appeared more similar to those of sterols, which likely suggests that GDN itself can occupy these sites. Our data suggest that while cholesterol may play an important role in the cellular context, its interaction with the TCR is insufficient to retain significant occupancy following solubilization and reconstitution.

During preparation of this work, cryo-EM structures of TCR complexes reconstituted in substantially smaller MSP1D1ΔH5 nanodiscs (~7 nm diameter) were reported<sup>48</sup>, describing conformational rearrangements within the core TCR upon pMHC binding. Comparison with our structures, together with our own reconstruction attempts using MSP1D1 (~10 nm diameter) nanodiscs, suggests that confined membrane environments may not fully preserve the native organization of the TCR transmembrane assembly (Fig. 5g,h). These observations align with increasing evidence that membrane constraints imposed by insufficiently sized nanodiscs can influence the structure and conformational landscape of embedded membrane proteins<sup>49-53</sup>. Consistent with this interpretation, larger MSP2N2 nanodiscs (~17 nm diameter) in our work supported higher-resolution complex structures, whereas TCR in detergent showed comparatively increased heterogeneity, and smaller MSP1D1 nanodiscs resulted in lower-resolution reconstructions with increased orientation bias (Fig. 5a-f).

The inability of cryo-EM to visualize intrinsically disordered cytosolic regions has long limited the mechanistic interpretation of TCR activation. By combining cryo-EM with smFRET, we show that structural rigidity of the receptor architecture is accompanied by antigen-induced rearrangement of CD3 $\zeta$ , thereby reconciling static structural observations with receptor activation. Cognate pMHC recognition at the extracellular face of a proteoliposome produces a ligand-specific increase in accessibility of the engineered CD3 $\zeta$  terminus on the opposite side of the membrane. The results are consistent with the allosteric release and detachment of membrane-associated cytosolic CD3 $\zeta$  domains upon pMHC binding<sup>26,54,55</sup>. Reconstitution of single TCR complexes and their engagement by individual, freely-diffusible pMHC molecules demonstrate that a single cognate receptor–ligand pair is sufficient to propagate this dynamic activation signature across the membrane.

The ability of soluble, monomeric pMHC to trigger the TCR has long been debated<sup>56-59</sup>. Our data show that cognate soluble pMHC is sufficient to generate a receptor-dependent CD3 $\zeta$ -accessibility signature in a defined system. Whether this signature is sufficient for cellular activation cannot be determined in the present assay. Additionally, antigen-induced CD3 $\zeta$  rearrangement was independent of liposomes containing single or multiple receptors. Cluster formation of TCRs has been widely discussed to play an important role in T cell activation<sup>21-23,60</sup>. While direct allosteric effects caused by pMHC recognition did not require the presence of multiple TCRs in our experiments, clustering may be necessary for further signal transduction and kinetic segregation of phosphatases<sup>19,20,61</sup>. In vivo, co-receptor recruitment, phosphatase exclusion, receptor organization, membrane composition, and mechanical inputs are likely to regulate the threshold and persistence of this response.

Our experiments characterize the effects of phosphorylation-dependent CD3 $\zeta$  modulation in comparison to ligand-induced effects. We show that Lck-mediated phosphorylation causes CD3 $\zeta$  ensemble shifts comparable to those triggered by cognate pMHC. Consistent with previous observations, negative charges added by phosphorylation increase repulsion with acidic membrane lipid headgroups<sup>28,55,62</sup>. Furthermore, we observed an additive effect between both signals, in agreement with ligand recognition and phosphorylation converging on a shared cytosolic conformational ensemble. Kinases likely amplify and reinforce ligand-induced CD3 $\zeta$  rearrangements during physiological activation, requiring multiple signals to cause T cell activation. However, the present data do not establish the temporal order of these events, direct membrane dissociation of CD3 $\zeta$ , or a positive-feedback mechanism. Resolving these questions

will require time-resolved phosphorylation measurements and orthogonal assays of CD3 $\zeta$  membrane proximity.

Integrating our structural and single-molecule analyses, we propose a working model in which cognate pMHC recognition initiates a dynamic cytosolic response without requiring large-scale rearrangements of the TCR core (Fig. 6). Higher binding affinities exhibited by antigenic pMHCs over self-pMHCs modulate the stability of the interaction rather than inducing conformational changes within the TCR. Stable pMHC/TCR complexes in a conformational equilibrium function as rigid structures and experience diffusional slowdown relative to unliganded receptor complexes, leading to destabilization of the association between CD3 $\zeta$  and the inner T cell membrane. Longer TCR dwell times at cognate pMHC molecules promote recruitment of the co-receptor to the immunological synapse. Previously spatially separated from the TCR, Lck is then available to begin phosphorylation of ITAMs. Clustering of TCRs, kinetic segregation of phosphatases, and recruitment of activating kinases may play an important role to stabilize the phosphorylated state of CD3 $\zeta$  in living cells. While the core TCR remains structurally unchanged, allosteric CD3 $\zeta$  membrane dissociation causes increasing cytosolic accessibility of CD3 $\zeta$ . Lck-mediated phosphorylation then becomes more efficient, which in turn further amplifies the shift in the CD3 $\zeta$  population. Finally, a complete shift of the CD3 $\zeta$  conformational ensemble towards the cytosol renders it fully accessible to cytosolic kinases, transforming the activated TCR into a signaling hub. Subsequent binding and activation of ZAP-70 facilitates further signal propagation, which eventually culminates in T cell activation.

Together, our work identifies a dynamic signaling layer that reconciles a structurally conserved TCR core with a ligand-responsive cytosolic output. Rather than functioning solely through a large-scale static structural switch, the TCR can couple cognate pMHC recognition to redistribution of CD3 $\zeta$  accessibility within an intrinsically disordered signaling ensemble. This framework provides a tractable basis for defining how membrane association, phosphorylation, force, receptor organization, and phosphatase control are integrated during T cell activation.

## Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.25716/gude.0rwv-tvxg>.

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## Figures

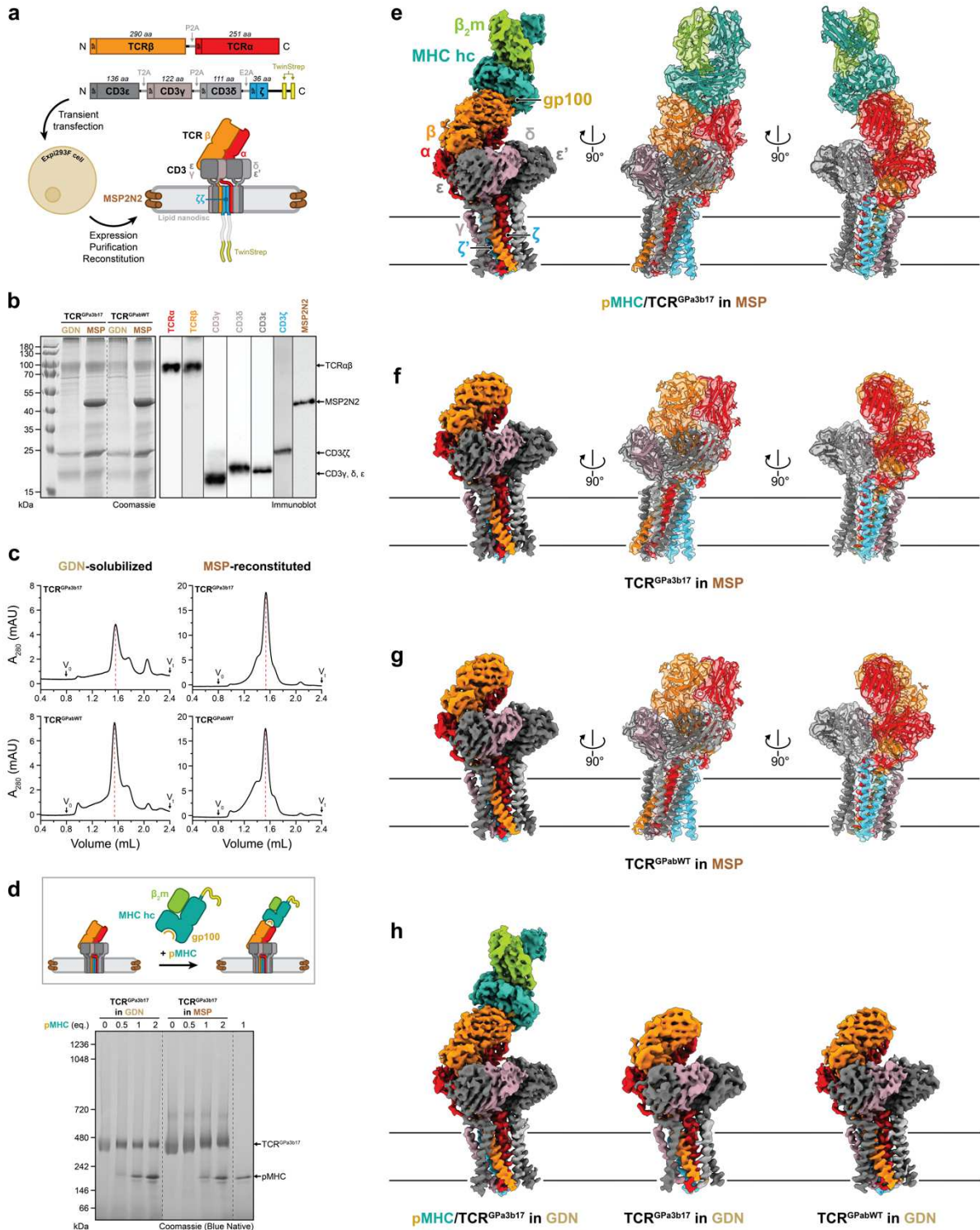


**Fig. 1 | Membrane-embedded human TCR complexes adopt a conserved architecture across ligand states and environments. a–d,** Biochemical validation of intact TCR complex reconstitution. **a,** Schematic of recombinant expression and reconstitution strategy. TCRβ $\alpha$  and CD3 $\epsilon\gamma\delta\zeta$  subunits were co-expressed in Expi293F cells by transient transfection. Signal

peptides (SP), self-cleaving peptides (P2A, T2A, E2A), and the C-terminal Twin-Strep affinity tag fused to CD3 $\zeta$  are indicated. Affinity-purified TCR complexes are reconstituted into MSP2N2 lipid nanodiscs. **b**, Purity and subunit composition of isolated TCR complexes in GDN detergent micelles and MSP2N2 lipid nanodiscs assessed by non-reducing SDS-PAGE with Coomassie staining or immunoblotting using subunit-specific antibodies. **c**, Size-exclusion chromatography profiles of TCR complexes in GDN detergent micelles and MSP2N2 lipid nanodiscs demonstrate formation of homogeneous, fully assembled receptor complexes in both environments. **d**, Blue Native PAGE analysis showing ligand-dependent mobility shift upon addition of cognate gp100/HLA-A\*02:01 pMHC, demonstrating preservation of ligand-binding activity after purification and membrane reconstitution. **e–h**, Cryo-EM structures of fully assembled human TCR complexes reveal the architecture of the intact receptor in membrane and detergent environments. **e**, Density map and atomic model of the affinity-enhanced TCR<sup>GPa3b17</sup> complex reconstituted into MSP2N2 (MSP, ~17 nm diameter) lipid nanodiscs and bound to cognate gp100/HLA-A\*02:01 (pMHC). Subunit identities are indicated and color-coded consistently throughout the figure. **f**, Apo affinity-enhanced TCR<sup>GPa3b17</sup> complex reconstituted in MSP2N2 lipid nanodiscs. **g**, Apo parental wild-type TCR<sup>GPa<sup>b</sup>WT</sup> complex reconstituted in MSP2N2 lipid nanodiscs. **h**, Cryo-EM density maps of affinity-enhanced and wild-type TCR complexes solubilized in GDN detergent micelles. See also [Extended Data Fig. 1–4](#).

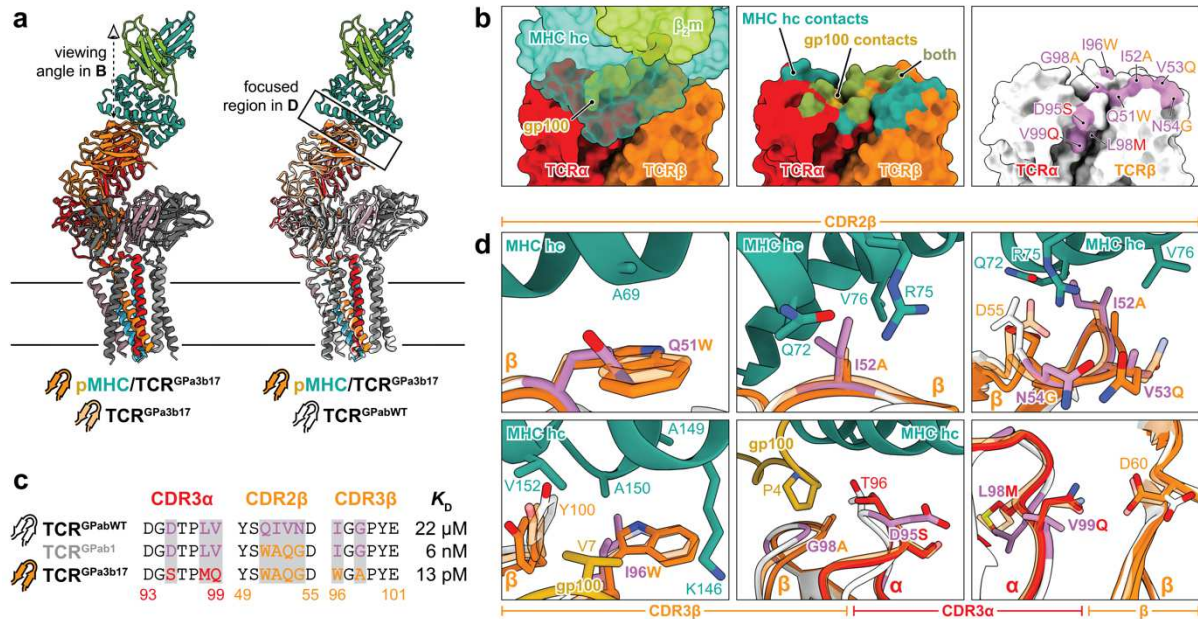


**Fig. 2 | Ligand affinity is modulated by localized remodeling of the pMHC-binding interface caused by individual amino acid substitutions.** **a**, Structural superposition of pMHC-bound affinity-enhanced TCR<sup>GPa3b17</sup> with apo affinity-enhanced TCR<sup>GPa3b17</sup> (left) and apo parental TCR<sup>GPaWT</sup> (right), both reconstituted in MSP2N2 lipid nanodiscs (~17 nm diameter). See also [Extended Data Fig. 1](#). **b**, Structural mapping of affinity-enhancing substitutions onto the TCR antigen-binding apex. Left, overall organization of the pMHC-binding surface. Middle, footprint of the TCR–pMHC interaction. Right, positions and identities of affinity-enhancing substitutions, illustrating that mutations cluster within the antigen-binding interface. **c**, Comparison of the complementarity-determining region (CDR) sequences from the parental receptor TCR<sup>GPaWT</sup>, an intermediate affinity-enhanced variant TCR<sup>GPa1</sup>, and the fully affinity-enhanced receptor TCR<sup>GPa3b17</sup><sup>34</sup>. Amino acid substitutions introduced during affinity enhancement are highlighted in purple together with the corresponding equilibrium dissociation constants ( $K_D$ ) for pMHC binding. Nine substitutions across CDR3 $\alpha$ , CDR2 $\beta$ , and CDR3 $\beta$  distinguish TCR<sup>GPaWT</sup> from TCR<sup>GPa3b17</sup>, increasing the affinity from 22  $\mu$ M to 13 pM. Residue numbering is indicated for reference. Notably, four mutations in CDR2 $\beta$  already account for a  $\sim 10^3$ -fold increase in affinity of the intermediate TCR<sup>GPa1</sup>. **d**, Close-up views of the three-way structural superposition of pMHC-bound affinity-enhanced TCR<sup>GPa3b17</sup> (colored), unliganded (apo) TCR<sup>GPa3b17</sup> (semi-transparent), and TCR<sup>GPaWT</sup> (white; affinity-enhanced residues highlighted in purple). Several substitutions appear to optimize local packing at the interface. CDR2 $\beta$  Q51W and I52A reduce potential steric clashes and promote a flatter hydrophobic binding interface, whereas CDR3 $\beta$  I96W and G98A increase local packing and peptide enclosure. Other substitutions appear to act indirectly.

The CDR2 $\beta$  loop containing V53Q and N54G does not form obvious direct contacts with pMHC, suggesting that these changes may instead influence solvent interaction or loop conformational dynamics. Similarly, CDR3 $\alpha$  substitutions remodel the loop backbone and stabilize the TCR $\alpha\beta$  framework rather than making extensive direct contacts with pMHC.

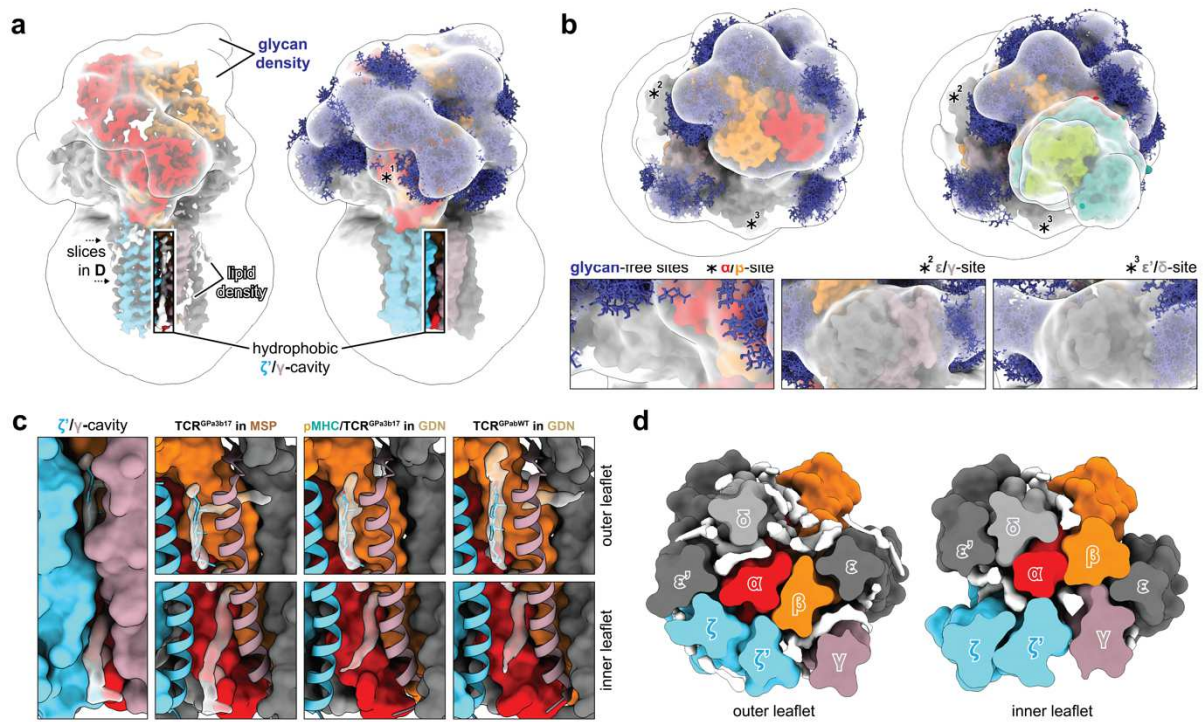


**Fig. 3 | Glycans and lipids shape the TCR architecture and pMHC recognition surface.**

**a**, High-resolution cryo-EM density map (left) and molecular model (right) of apo TCR<sup>GPa3b17</sup> (colored) reconstituted in MSP2N2 lipid nanodiscs, displayed within the corresponding low-pass filtered density envelope (transparent white), highlighting flexible peripheral regions. Lipid densities within the transmembrane region are shown as white isosurfaces (left), whereas resolved N-linked glycans are shown as blue stick representations (right). **b**, Comparison of low-pass filtered cryo-EM density maps for apo (left) and pMHC-bound (right) TCR<sup>GPa3b17</sup> viewed from the extracellular side. The EM map closely matches the conformational space sampled by the MD-derived glycan ensemble (blue sticks, see [Online Methods](#)), revealing extensive glycan shielding of the receptor surface while preserving accessibility of the pMHC-binding apex. Additional glycan-free regions are observed adjacent to the membrane and at both CD3 $\epsilon$  subunits (asterisks). **c**, Comparison of the canonical cholesterol-binding site between CD3 $\zeta'$  and CD3 $\gamma$  in nanodisc- and detergent-reconstituted complexes. Lipid densities are better resolved in large nanodiscs, suggesting that this conserved pocket can accommodate different types of membrane lipids. CD3 $\gamma$  and CD3 $\zeta'$  are shown as space-filling model (left) and in ribbon representation (right) to visualize the associated densities within the outer and inner membrane leaflets. **d**, Cutaway views of the transmembrane assembly illustrating the distribution of tightly intercalated lipid densities associated with the outer (left) and inner (right) membrane leaflets. Proteins are shown as space-filling models to emphasize the tight association of non-annular lipids with the TCR transmembrane assembly.

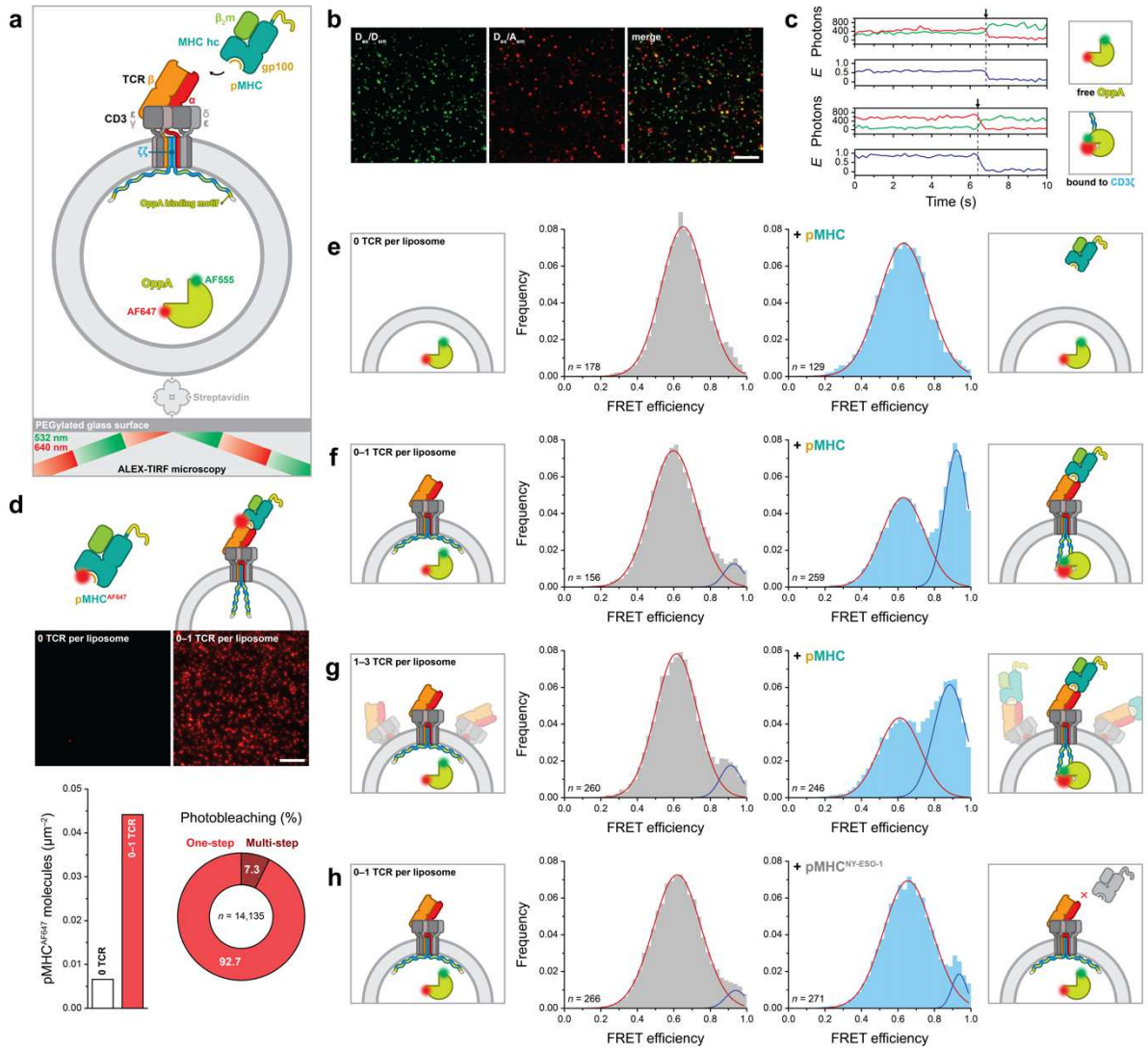


**Fig. 4 | pMHC recognition triggers ligand-specific rearrangement of the CD3 $\zeta$  conformational ensemble.** **a**, Schematic of the single-molecule FRET assay used to monitor changes in CD3 $\zeta$  accessibility within fully assembled TCR complexes. Proteoliposomes containing reconstituted TCR complexes with full-length CD3 $\zeta$  (ITAMs shown in yellow) and encapsulated FRET sensor (OppA) were immobilized on PEGylated glass surfaces via biotinylated lipids linked to streptavidin. Soluble ligands, including pMHC, were added to the exterior of the liposomes, and binding-induced conformational changes of OppA were monitored by alternating laser excitation (ALEX) total internal reflection fluorescence (TIRF) microscopy. **b**, Representative fluorescence micrographs showing donor emission upon donor excitation ( $D_{ex}/D_{em}$ ), acceptor emission upon donor excitation ( $D_{ex}/A_{em}$ ), and a merged image highlighting colocalized donor and acceptor signals (scale bar 5  $\mu m$ ). **c**, Representative single-molecule fluorescence trajectories of OppA in low- and high-FRET states. Photon counts and corresponding FRET efficiencies are shown. Acceptor photobleaching events confirming

single-molecule detection are indicated by arrows and dashed lines. **d**, Incorporation of single TCRs was verified by photobleaching experiments of liposomes stained with pMHC<sup>AF647</sup>. Representative fluorescence micrographs of liposomes containing no (0) or individual (0–1) TCR complexes (top) illustrate low unspecific surface binding of pMHC<sup>AF647</sup>. Signal densities in each measurement are quantified in the bar chart (bottom left). Nearly all liposomes showed one-step photobleaching (bottom right), indicating that no more than one TCR was reconstituted per liposome (scale bar 5  $\mu$ m). **e–h**, OppA FRET efficiency distribution before and after ligand addition. **e**, liposomes following addition of cognate pMHC. **f**, Proteoliposomes containing individual (0–1) TCR complexes following addition of cognate pMHC. **g**, Proteoliposomes containing multiple (1–3) TCR complexes following addition of cognate pMHC. **h**, Proteoliposomes containing individual (0–1) TCR complexes following addition of non-cognate pMHC<sup>NY-ESO-1</sup>. Cognate antigen selectively shifted the CD3 $\zeta$  ensemble toward a high-FRET state independent of receptor clustering, whereas non-cognate pMHC failed to induce this transition. Data represent  $N = 3$  independent experiments. See also [Extended Data Fig 6 and 7](#).

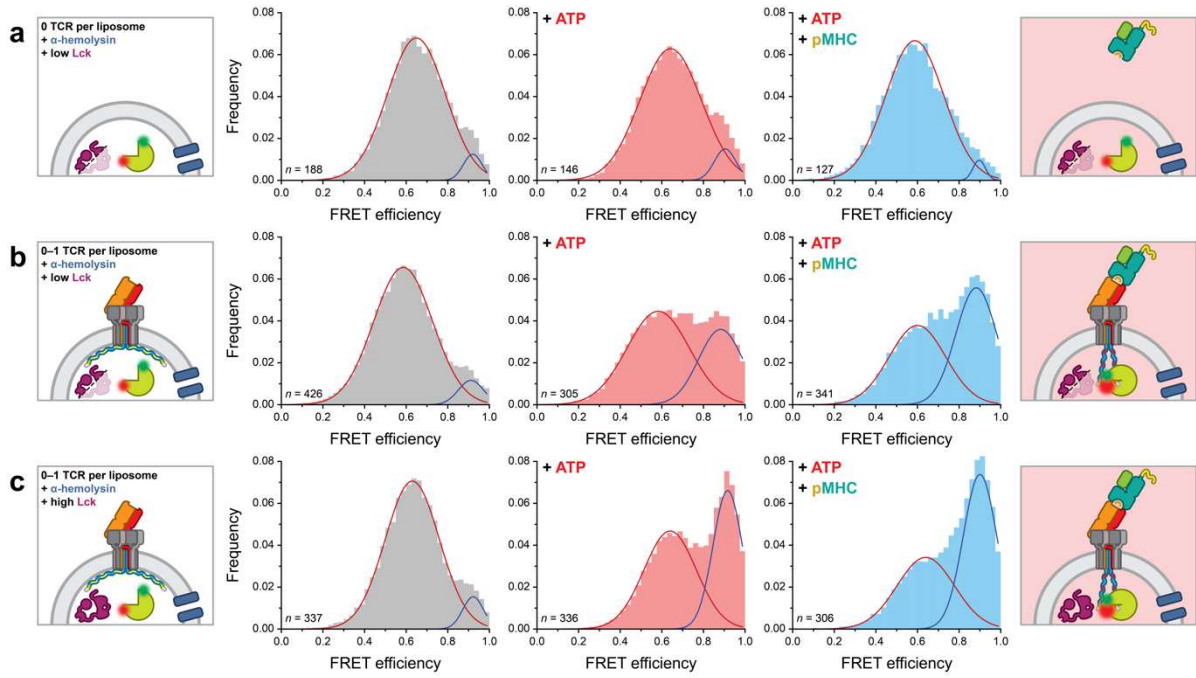


**Fig. 5 | Phosphorylation by Lck is complementary to antigen recognition in driving CD3 $\zeta$  ensemble rearrangement.** OppA FRET efficiency distributions following sequential addition of ATP (red background) and cognate pMHC under different reconstitution conditions (phosphorylated ITAMs shown in red). **a**, Liposomes with Lck lacking TCR complexes serve as a control for TCR-dependent signaling responses. **b**, Proteoliposomes containing individual TCR complexes at sub-stoichiometric Lck occupancy. ATP addition induces a partial shift toward higher FRET states, consistent with phosphorylation-dependent increased accessibility of CD3 $\zeta$ , whereas subsequent cognate pMHC engagement produces an additional transition toward the high-FRET state. **c**, Proteoliposomes containing individual TCR complexes at stoichiometric Lck occupancy. Here, phosphorylation is sufficient to induce transition to a similar high-FRET as observed for pMHC binding alone. Recognition of pMHC further increases CD3 $\zeta$  accessibility beyond previous experiments. Data represent  $N = 3$  independent experiments.

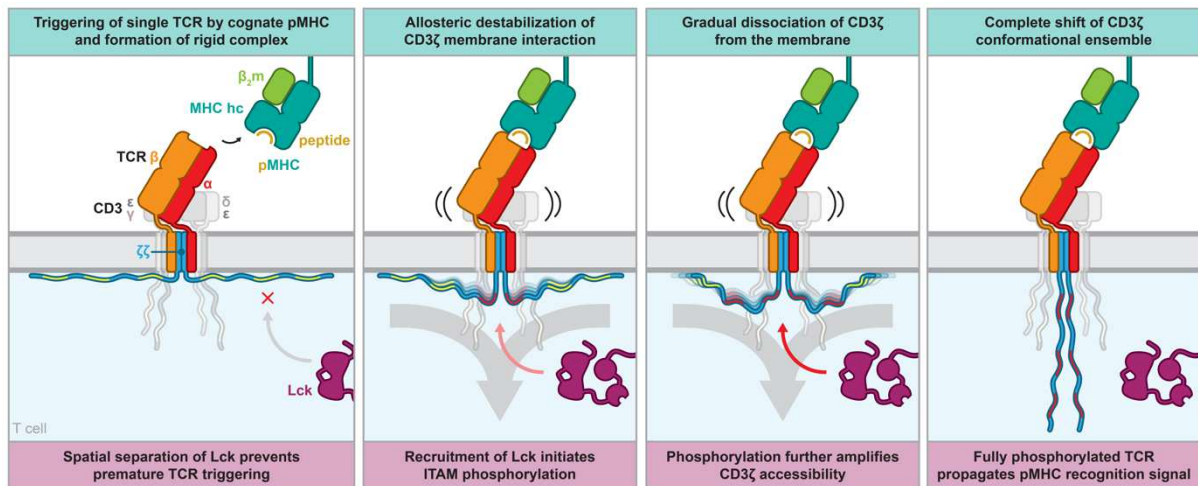


**Fig. 6 | Signal transduction by the TCR requires cognate pMHC recognition as well as Lck-mediated phosphorylation.** Schematic illustration of a TCR triggering model suggested by our data (ITAMs shown in yellow). Antigenic pMHC forms a stable complex with a single TCR, acting as a rigid body to cause destabilization of the membrane association of CD3 $\zeta$ . Spatially separated Lck is now recruited to the TCR to initiate ITAM phosphorylation (phosphorylated ITAMs shown in red). The conformational ensemble shifts towards the cytosol to become fully accessible to kinase activity, amplifying subsequent signal propagation. The specific activity of Lck increases due to greater accessibility as CD3 $\zeta$  dissociates from the membrane, working in tandem to modulate the T cell response.

## Online Methods

### TCR expression in Expi293F cells

The gp100-specific parental TCR (TCR<sup>GPabWT</sup>) is encoded by several gene segments<sup>34</sup>: The TCR $\alpha$  V domain is encoded by TRAV17\*01 and the C region by TRAC\*01; the TCR $\beta$  V domain is encoded by TRBV19\*01 and the C region by TRBC1\*01. Amino acid substitutions introduced during affinity maturation to generate TCR<sup>GPab17</sup> are summarized in [Fig. 2](#).

For co-expression of the complete human TCR complex, two multicistronic expression plasmids were generated ([Fig. 1e](#)). The TCR $\beta\alpha$  plasmid encoded the full-length TCR $\beta$  (residues 1–310) and TCR $\alpha$  (residues 1–270), preceded by the signal peptides MSLSSLLKVVVTASLWLGPPI (TCR $\beta$ ) and MGFRLCCVAFCLLGAGPV (TCR $\alpha$ ). The two chains were separated by a furin cleavage site, a GSG linker, and a P2A ribosome-skipping sequence<sup>15</sup>.

The CD3 $\epsilon\gamma\delta\zeta$  plasmid encoded the C-terminally truncated subunits CD3 $\epsilon$  (residues 1–158), CD3 $\gamma$  (residues 1–144), CD3 $\delta$  (residues 1–132), CD3 $\zeta$  (residues 1–57), separated by GSG linkers and successive T2A, P2A, and E2A ribosome-skipping sequences. For purification, CD3 $\zeta$  was C-terminally fused to a TwinStrep tag via a 42 amino acid linker. In the TCR<sup>QAF</sup> construct, full-length CD3 $\zeta$  (residues 1–142) replaced the truncated variant and was extended by the OppA-binding sequence RDMPIQAF, derived from  $\beta$ -casein<sub>183–190</sub>, in which the N-terminal arginine corresponds to the native C-terminal residue (R142) of CD3 $\zeta$ . A tobacco etch virus (TEV) protease cleavage site was inserted between the OppA-binding sequence and the TwinStrep tag.

Expi293F cells (Gibco) were maintained in FreeStyle293 Expression Medium (Gibco) at 37 °C with 5% CO<sub>2</sub> and orbital shaking (125 rpm). Cells were transiently co-transfected using linear polyethylene imine (PEI, Polysciences) with plasmids encoding TCR $\beta\alpha$  and CD3 $\epsilon\gamma\delta\zeta$  at a ratio of 2:1 (w/w) and a DNA:PEI ratio of 1:3 (w/w). Plasmid DNA was prepared from *Escherichia coli* Mach1 T1 cells using the PureYield Plasmid Midiprep System (Promega).

For transfection, DNA (300  $\mu$ g) and PEI were incubated for 30 min in expression medium before addition to 100 mL Expi293F cultures at a density of 5–6  $\times 10^6$  cells/mL. Cultures were diluted to 500 mL with fresh medium after 3 h and incubated for a total of 72 h. Cells were harvested by centrifugation (500  $\times$  g, 10 min, 4 °C), washed once with ice-cold DPBS (Gibco), and processed immediately or stored at –80 °C.

### **TCR<sup>GPa3b17</sup>, TCR<sup>GPabWT</sup>, and TCR<sup>QAF</sup> purification**

Cell pellets from 500-mL cultures were resuspended in lysis buffer (30 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 1% (w/v) GDN (Anatrace), 1× Protease Inhibitor Mix HP (Serva)), lysed by sonication on ice, and treated with 0.01% (v/v) Benzonase Nuclease (10 kU/pc, Merck Millipore). The lysate was solubilized for 2 h by rotation at 8 °C. Insoluble material was removed by ultracentrifugation (142,000 × g, 1 h, 4 °C). Solubilized TCR complexes were purified by Strep-Tactin XT affinity chromatography (Iba). Complexes were bound overnight to 500 µL resin slurry by rotation at 8 °C. Resin was transferred to gravity-flow columns (Bio-Rad) and washed sequentially with TCR buffer (20 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 1 mM CaCl<sub>2</sub>) first containing 0.15% (w/v) GDN, then 0.05% (w/v) GDN and 5 mM biotin. Complexes were either eluted in TCR buffer containing 0.05% (w/v) GDN and 100 mM biotin or reconstituted directly in nanodiscs while bound to the affinity resin. Eluted complexes were concentrated (VivaSpin 500 (100,000 Da), Sartorius) by centrifugation (4,000 × g, 4 °C). Aggregates were removed by a final centrifugation step (21,300 × g, 5 min, 4 °C).

TCR<sup>QAF</sup> was purified as above with the following modifications. After binding, the resin was washed with TCR buffer first containing 0.15% (w/v) GDN and 5 mM biotin, then 0.05% (w/v) GDN. Resin-bound complexes were eluted by incubation overnight with TEV protease (20% (w/w) of expected yield) at 8 °C by affinity tag cleavage. TEV protease was removed by reverse Ni-NTA affinity chromatography (Qiagen) prior to concentration of the purified complex.

### **MSP preparation**

Plasmids encoding MSP2N2 and MSP1D1 were obtained from Stephen Sligar (Addgene plasmids #29520 and #20061). Membrane scaffold proteins (MSPs) were expressed and purified as previously described<sup>63</sup>. Briefly, *E. coli* T7 Express cells were transformed with respective plasmids and cultured in Terrific Broth supplemented with kanamycin (50 µg/mL) at 180 rpm and 37 °C. At an OD<sub>600</sub> of 0.8, protein expression was induced with IPTG (1 mM final concentration), and cultures were incubated for 4 h at 28 °C before harvesting. Cells pellets were stored at −80 °C.

For purification, pellets were resuspended in MSP buffer (40 mM Tris-HCl pH 8.0, 300 mM NaCl) supplemented with 1 mM MgCl<sub>2</sub>, 1 mM PMSF and 1% (v/v) Triton X-100. Cells were

lysed by sonication, treated with Benzonase Nuclease (0.01%, v/v), incubated for 20 min at 4 °C, and cleared by centrifugation ( $50,000 \times g$ , 30 min, 4 °C). MSPs were purified by Ni-NTA affinity chromatography (Qiagen) after binding to the resin for 2 h at 8 °C. The resin was washed sequentially with MSP buffer first containing 1% (v/v) Triton X-100, then 20 mM imidazole and 50 mM sodium cholate, and finally 50 mM imidazole. Proteins were eluted in MSP buffer supplemented with 500 mM imidazole.

Purified MSPs were exchanged into MSP buffer using 10DG desalting columns (Bio-Rad), concentrated (Amicon Ultra-4 (10,000 Da), Merck Millipore) by centrifugation ( $2,500 \times g$ , 4 °C), supplemented with 10% (v/v) glycerol, aliquoted, and stored at –80 °C.

### **Reconstitution of TCR complexes in lipid nanodiscs**

TCR complexes were reconstituted directly on the affinity resin into lipid nanodiscs assembled with MSP2N2 or MSP1D1 using optimized molar ratios of 1:5:500 (TCR:MSP2N2:lipid) or 1:5:200 (TCR:MSP1D1:lipid), based on the expected receptor yield. Bovine brain lipid extract (Sigma) was dried from chloroform under vacuum and resuspended at 20 mg/mL in lipid buffer (20 mM HEPES-NaOH pH 7.5, 150 mM NaCl). Lipids were solubilized with a two-fold molar excess of sodium cholate and incubated for 30 min at 4 °C before nanodisc assembly.

Resin-bound TCR complexes were incubated with solubilized lipids (12 mM final sodium cholate concentration) for 15 min at 8 °C with gentle rotation, followed by addition of MSPs and incubation for 30 min. Detergent was removed by sequential addition of Bio-Beads SM-2 (Bio-Rad) at 8 °C (40 mg for 1 h, followed by 80 mg overnight). The resin was transferred to gravity-flow columns, washed with TCR buffer, and nanodisc-reconstituted complexes were eluted, concentrated, and clarified as described above.

### **Peptide synthesis and labeling**

Peptides were synthesized by standard Fmoc-based solid-phase peptide synthesis (SPPS) on a Liberty Blue microwave peptide synthesizer (CEM) using Fmoc-protected Wang resin (Rapp Polymere). The resin was pre-swollen in dimethylformamide, and amino acids were coupled by double-coupling cycles using 0.2 M Fmoc-protected amino acids, 0.5 M *N,N'*-diisopropylcarbodiimide, and 1 M hydroxybenzotrazole hydrate in DMF. Fmoc groups were

removed with 20% (v/v) piperidine in DMF. After synthesis, the resin was washed sequentially with DMF, dichloromethane, methanol, chloroform, and diethyl ether. Finally, peptides were cleaved off the resin by incubation in trifluoroacetic acid:triisopropylsilane:H<sub>2</sub>O at a ratio of 95:2.5:2.5 (v/v) and precipitated in diethyl ether over night at −20 °C.

For fluorescent labeling, Fmoc-protected gp100K (Fmoc-YLEPGPVTVK) was reacted overnight at room temperature with N-hydroxysuccinimide (NHS)-activated Alexa Fluor 647 (AF647, Thermo Fisher Scientific) at a 3:1 dye-to-peptide molar ratio in dimethylsulfoxide containing 5% (v/v) *N,N*-diisopropylethylamine. The labeled peptide was purified by C18 reverse-phase high-performance liquid chromatography (RP-HPLC, PerfectSil 300 ODS, 5 µm, 250 × 10 mm, MZ-Analysentechnik) using a linear acetonitrile gradient (5–80% in 20 min) supplemented with 0.1% (v/v) formic acid, deprotected by Fmoc removal, and again purified by C18 RP-HPLC before analysis by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS).

### **pMHC preparation**

Human  $\beta_2$ -microglobulin ( $\beta_2m$ ) and the HLA-A\*02:01 heavy chain (MH hc) were produced and refolded as previously described<sup>64</sup>. Briefly, *E. coli* BL21(DE3) cells were transformed with respective plasmids and cultured in LB medium supplemented with ampicillin (100 µg/mL for  $\beta_2m$ ) or kanamycin (50 µg/mL for MHC hc) at 37 °C under shaking (180 rpm). At an OD<sub>600</sub> of 0.6, protein expression was induced with IPTG (1 mM final concentration), and cultures were incubated for 4 h at 37 °C before harvesting. Cells pellets were stored at −80 °C.

For inclusion body isolation, cells pellets were resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 2 mM MgCl<sub>2</sub>, 10 mM DTT, 1% (v/v) Triton X-100, Protease Inhibitor Mix HP (Serva)). Cells were lysed by sonication, treated with Benzonase Nuclease (0.01%, v/v)), and incubated for 15 min at 4 °C. Inclusion bodies were isolated by seven rounds of centrifugation (10,000 × g, 20 min, 4 °C) and resuspension in inclusion body buffer (20 mM Tris-HCl pH 8.0, 2.5 mM EDTA), including five washes containing 0.5% (v/v) Triton X-100 followed by two detergent-free washes. Inclusion bodies were stored at −80 °C until refolding.

$\beta_2m$  inclusion bodies were pelleted (20,000 × g, 5 min, 4 °C) and solubilized for 1 h at 8 °C in buffer (100 mM Tris-HCl pH 8.0, 8 M urea, and Protease Inhibitor Mix HP (Serva)) with gentle rotation. Insoluble material was removed by centrifugation (16,000 × g, 5 min, 4 °C).  $\beta_2m$  was

refolded by dialysis against 10 mM Tris-HCl pH 8.0 using Spectra/Por 7 dialysis tubing (3,500 Da, Carl Roth). Refolded protein was concentrated (Amicon Ultra-15 (3,000 Da), Merck Millipore) by centrifugation ( $4,000 \times g$ , 4 °C), exchanged into SEC buffer (10 mM HEPES-NaOH pH 7.5, 150 mM NaCl) using Zeba Spin desalting columns (Thermo Fisher Scientific), and purified by SEC (HiLoad 16/60 Superdex 75 pg, Cytiva). Purified  $\beta_2m$  was aliquoted and stored at  $-80$  °C.

MHC hc inclusion bodies were pelleted ( $10,000 \times g$ , 10 min, 4 °C) and solubilized overnight at 8 °C in buffer (20 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 6 M guanidine hydrochloride). Insoluble material was removed by centrifugation ( $30,000 \times g$  30 min, 4 °C). Solubilized MHC hc (1  $\mu$ M final concentration) was refolded by rapid dilution into refolding buffer (100 mM Tris-HCl pH 8.0, 2 mM EDTA, 400 mM L-arginine-HCl, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, and Protease Inhibitor Mix HP (Serva)) containing 2  $\mu$ M  $\beta_2m$  and either 40  $\mu$ M gp100<sub>280-288</sub><sup>A288V</sup> (YLEPGPVTV) peptide, 40  $\mu$ M NY-ESO-1<sub>157-165</sub><sup>C165V</sup> peptide (SLLMWITQV), or 10  $\mu$ M gp100K<sup>AF647</sup> peptide (YLEPGPVTVK<sup>AF647</sup>). Refolding reactions were stirred for 96 h at 8 °C and concentrated (Amicon Ultra-15 (30,000 Da), Merck Millipore) by centrifugation ( $4,000 \times g$ , 4 °C).

Refolded pMHC complexes were purified by size-exclusion chromatography (Superdex 200 Increase 10/300 GL, Cytiva) in SEC buffer. Peak fractions were pooled, concentrated (Amicon Ultra-0.5 (30,000 Da), Merck Millipore) by centrifugation ( $4,000 \times g$ , 4 °C), flash-frozen in aliquots, and stored at  $-80$  °C.

### smFRET sensor preparation

Fluorescently labeled *Lactococcus lactis* OppA<sup>A209C/S441C</sup> (LlOppA<sup>AS</sup>) was prepared as previously described<sup>37</sup>. *E. coli* MC1061 cells were transformed with corresponding plasmid DNA and cultured in Terrific Broth supplemented with ampicillin (100  $\mu$ g/mL) at 37 °C under shaking (180 rpm). At an OD<sub>600</sub> of 0.4, protein expression was induced with arabinose (0.1% (w/v) final concentration), and cultures were incubated overnight at 25 °C before harvesting. Cells pellets were stored at  $-80$  °C.

For purification, pellets were resuspended in lysis buffer (25 mM potassium phosphate (KP<sub>i</sub>) pH 6.5, 100 mM KCl, 10% (v/v) glycerol, 15 mM imidazole, 1 mM MgSO<sub>4</sub>, 0.5 mM PMSF, 1 mM DTT). Cells were lysed by sonication and cleared by centrifugation ( $18,000 \times g$ , 35 min,

4 °C). *L/OppA<sup>AS</sup>* was purified by Ni-NTA affinity chromatography (Cytiva) in gravity flow columns. Resin was washed sequentially with OppA wash buffer (25 mM KP<sub>i</sub> pH 6.5, 100 mM KCl, 10% (v/v) glycerol, 1 mM MgSO<sub>4</sub>, 1 mM DTT) first containing 15 mM, then 30 mM imidazole. Proteins were eluted in elution buffer (25 mM KP<sub>i</sub> pH 6.0, 20 mM KCl, 10% (v/v) glycerol, 400 mM imidazole, 1 mM MgSO<sub>4</sub>, 1 mM DTT) before further purification by cation exchange chromatography (HiTrap SP HP, Cytiva) using a linear KCl gradient (20–500 mM) in buffer (25 mM KP<sub>i</sub> pH 6.0, 10% (v/v) glycerol, 1 mM DTT).

To remove endogenously bound peptides, *L/OppA<sup>AS</sup>* was partially unfolded and refolded by stepwise dialysis at 8 °C in OppA refolding buffer (25 mM KP<sub>i</sub> pH 6.5, 100 mM KCl, 10% (v/v) glycerol, 1 mM DTT), containing decreasing concentrations of guanidine hydrochloride (2 M for 1 h, 1.5 M for 30 min, 1.0 M for 30 min, and 0.5 M for 30 min). Protein was concentrated (Amicon Ultra-0.5 (10,000 Da), Merck Millipore) by centrifugation (10,000 × g, 4 °C), and DTT was removed by Zeba spin desalting columns (Thermo Fisher Scientific).

Purified *L/OppA<sup>AS</sup>* was labeled overnight at 8 °C with maleimide-conjugated Alexa Fluor 555 (AF555, Thermo Fisher Scientific) and Alexa Fluor 647 at a dye-to-protein molar ratio of 10:10:1 in labeling buffer (25 mM HEPES-NaOH pH 7.5, 300 mM NaCl, 10% (v/v) glycerol). Residual maleimide groups were quenched with β-mercaptoethanol (1 mM final concentration), and excess fluorophores were removed by SEC (Superdex 200 Increase 3.2/300, Cytiva). Fluorescently labeled *L/OppA<sup>AS</sup>* (OppA) was stored at 4 °C.

### **Liposome preparation**

Lipid mixtures consisting of 40% POPC, 30% POPG, 29% POPE, and 1% biotinylated DOPE (w/v) were prepared from chloroform stocks at a total lipid concentration of 5 mg/mL. Solvent was removed by rotary evaporation, and the dried lipid film was hydrated in liposome buffer (25 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 5% (v/v) glycerol) to a final concentration of 5 mg/mL. The suspension was sonicated for 20 min and subjected to five freeze-thaw cycles to generate large unilamellar vesicles (LUVs) before storage at –80 °C. Right before proteoliposome reconstitution, LUVs were extruded through 100 nm polycarbonate membranes (Cytiva) using a LiposoFast-Basic extruder (Avestin) with 11 consecutive passes.

## TCR reconstitution in proteoliposomes

Stochastic reconstitution was utilized to obtain proteoliposomes containing desired numbers of molecules within a Poisson occupancy distribution. For reconstitution of TCR<sup>QAF</sup>, a mass ratio of 1:364 between TCR<sup>QAF</sup> and liposomes was assumed. Receptor-to-lipid mass ratios of 0.4:364 and 2:364 were applied to stochastically reconstitute 0–1 TCR<sup>QAF</sup> (mean occupancy 0.3) and 1–3 TCR<sup>QAF</sup> (mean occupancy 0.9) per liposome, respectively. The smFRET sensor OppA was encapsulated at a protein-to-liposome molar ratio of 0.2:1 (mean occupancy 0.2) to achieve optimal signal density. Lck in low and high concentrations was encapsulated at protein-to-liposome molar ratios of 1:1 (mean occupancy 0.6) and 4:1 (mean occupancy 1), respectively.

Liposomes were destabilized with 0.2% (v/v) Triton X-100 for 30 min before addition of GDN-solubilized TCR<sup>QAF</sup> and OppA, followed by incubation for 30 min at 8 °C with gentle rotation. Detergent was removed by sequential addition of Bio-Beads SM-2 at 8 °C (40 mg for 1 h, 40 mg overnight, 80 mg for 1 h, 80 mg for 1 h).

Proteoliposomes were collected by ultracentrifugation (300,000 × g, 30 min, 4 °C) and resuspended in imaging buffer (25 mM HEPES-NaOH pH 7.2, 150 mM NaCl, 5% (v/v) glycerol, 90 mM glucose, 5 mM Trolox, 7.5 U/mL pyranose oxidase, and 1 kU/mL catalase) for smFRET imaging.

## PAGE and immunoblot analysis

Blue and Clear Native PAGE were performed using NativePAGE 3–12% Bis-Tris gels (Thermo Fisher Scientific) according to manufacturer's instructions. NativeMark Unstained Protein Standard (Invitrogen) was used as a molecular size marker. For Blue Native PAGE, TCR<sup>GPa3b17</sup> in MSP complexes at 5 μM were incubated with increasing concentrations of pMHC for 30 min at 4 °C. For Clear Native PAGE, pMHC<sup>AF647</sup> at 100 nM was incubated with increasing concentrations of TCR<sup>QAF</sup> in GDN for 30 min at 4 °C.

SDS-PAGE analysis was performed with 13% Tris-glycine polyacrylamide gels. PageRuler Prestained Protein Standard (Thermo Fisher Scientific) was used as a molecular size marker. For immunoblots (Fig. 1f), samples of TCR<sup>GPa3b17</sup> in MSP were analyzed under non-reducing (TCRα, TCRβ, CD3ζ and MSP2N2) or reducing (CD3γ, CD3δ, CD3ε) conditions. Proteins were visualized by Coomassie staining with InstantBlue (Abcam) or transferred to

nitrocellulose membranes (Cytiva) by a Trans-Blot Turbo transfer system (Bio-Rad). Membranes were blocked for 1 h in DPBS containing 5% (w/v) milk powder or biotin-free BSA in case of streptavidin-HRP detection. Primary antibodies (TCR $\alpha$ , TCR $\beta$ , CD3 $\delta$ , CD3 $\epsilon$ , CD3 $\zeta$  at 1:1,000; CD3 $\gamma$ , poly-His at 1:3,000) or streptavidin-HRP (1:20,000) were applied at 8 °C overnight. After three washes in DPBS containing 0.1% (v/v) Tween20, membranes were incubated with secondary antibodies (1:20,000) for 1 h. Immunoreactive bands were detected by chemiluminescence using Clarity Western ECL Substrate (Bio-Rad) and a Fusion FX imaging system (Vilber).

### **SEC analysis**

Analytical SEC was performed in TCR buffer supplemented with 0.05% (v/v) GDN for detergent-solubilized TCR complexes (Superose 6 Increase 3.2/300; Cytiva). Samples were prepared by centrifugation ( $21,300 \times g$ , 10 min, 4 °C) prior to injection.

### **Cryo-EM sample preparation and data collection**

For ligand-bound structures, purified TCR complexes were incubated with pMHC at a 1:1 molar ratio for 30 min at 4 °C. Samples were then concentrated (VivaSpin 500 (100,000 Da)) by centrifugation ( $4,000 \times g$ , 4 °C) and clarified ( $21,300 \times g$ , 5 min, 4 °C).

Cryo-EM grids were prepared by applying 3  $\mu$ L of purified protein complex to glow-discharged Quantifoil R1.2/R1.3 300 mesh gold/gold grids. Samples were blotted for 5 s and plunge-frozen in liquid ethane using a Vitrobot Mark IV (Thermo Fisher Scientific) at 4 °C and 100% humidity.

Cryo-EM data were collected on a 200 kV Glacios transmission electron microscope (Thermo Fisher Scientific) equipped with a Falcon 3EC direct electron detector (Thermo Fisher Scientific) in counting mode. Dose-fractionated micrograph movies were recorded at a nominal magnification of 150,000 $\times$ , corresponding to a calibrated pixel size of 0.95 Å, with a dose rate of 0.8 e<sup>-</sup>/pixel/s for a total exposure time of 33.35 s distributed over 24 frames, resulting in a cumulative dose of  $\sim 28$  e<sup>-</sup>/Å<sup>2</sup> ([Extended Data Table 1](#)).

## Cryo-EM image processing

Cryo-EM datasets were processed in cryoSPARC (ref.<sup>65</sup>) (see [Extended Data Fig. 2–5](#)). Following motion correction and contrast transfer function (CTF) estimation, particles were identified using TOPAZ (ref.<sup>66</sup>). Initial particle stacks were extracted using three-fold Fourier cropping (2.85 Å/pixel) and subjected to multi-class *ab initio* reconstruction. Classes exhibiting well-defined protein-like densities were selected for iterative heterogeneous refinement to enrich high-quality TCR particles.

Selected particles were re-extracted at the original pixel size (0.95 Å pixel<sup>-1</sup>) and subjected to non-uniform refinement, reference-based motion correction, and CTF refinement, followed by a final round of non-uniform refinement. This workflow yielded six final cryo-EM reconstructions with global resolutions ranging from 2.72 Å to 3.81 Å ([Extended Data Table 1](#)). Across all datasets, nanodisc-reconstituted TCR complexes consistently produced higher-resolution reconstructions compared with detergent-solubilized preparations.

For visualization, model building, and coordinate deposition, final maps with resolutions better than 3.0 Å were additionally processed using phenix.auto\_sharpen (ref.<sup>67</sup>) to improve map interpretability.

## Molecular modeling and refinement

The previously reported detergent-solubilized pMHC/TCR structure (PDB: 7PHR) was used as a template for model building. Initial models were rigid-body fitted into cryo-EM density maps using UCSF ChimeraX (ref.<sup>68</sup>), followed by manual rebuilding in Coot (ref.<sup>69</sup>). Real-space refinement was performed in Phenix using phenix.real\_space\_refine (ref.<sup>67</sup>). For generation of the parental TCR<sup>GPa3b17</sup> model, affinity-enhancing substitutions present in TCR<sup>GPa3b17</sup> were reverted to the corresponding wild-type residues. Model refinement and validation statistics are summarized in [Extended Data Table 1](#).

## MD analysis of glycan conformations

To assess glycan shielding of the TCR ectodomains, experimentally assigned N-linked glycans were subject to MD simulations using the program GlycoShield (ref.<sup>70</sup>). Here, the glycan Man<sub>5</sub>GlcNAc<sub>2</sub>Fuc<sub>1</sub> was chosen as a baseline for glycan shielding. Simulated glycan

conformational ensembles were compared with low-pass-filtered cryo-EM density maps to evaluate agreement between predicted glycan flexibility and experimentally observed density envelopes.

### **Liposome incorporation of TCR<sup>QAF</sup>**

A liposome flotation assay was performed to assess incorporation of TCR<sup>QAF</sup> into liposomes. Proteoliposomes containing 1–3 TCR<sup>QAF</sup> complexes were added onto a discontinuous sucrose density gradient consisting of layers of 10%, 20%, 30% and 40% sucrose and centrifuged ( $300,000 \times g$ , 1 h, 4 °C). Gradient fractions were collected and analyzed by non-reducing SDS-PAGE and immunoblotting.

### **Orientation of TCR<sup>QAF</sup> in liposomes**

The orientation of reconstituted TCR<sup>QAF</sup> complexes was determined by a protease protection assay. Proteoliposomes containing 1–3 TCR<sup>QAF</sup> complexes were incubated with either liposome buffer (25 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 5% (v/v) glycerol), buffer supplemented with proteinase K (50 µg/mL, Thermo Fisher Scientific), or buffer containing both proteinase K (50 µg/mL) and Triton X-100 (1% (v/v)). Samples were incubated at 30 °C for 15 min, followed by protease inactivation with Pefabloc SC (5 mM, Roche) at 4 °C for 5 min. Proteins were precipitated by addition of trichloroacetic acid (TCA, 10% (v/v) final concentration) and incubation at 4 °C for 30 min. Precipitated proteins were washed with acetone by centrifugation ( $12,000 \times g$ , 15 min, 4 °C), dried at 37 °C for 5 min, and analyzed by non-reducing SDS-PAGE and immunoblotting.

### **Fluorescence lifetime measurements**

Fluorescence lifetime measurements were performed by time-correlated single photon counting (TCSPC) on a Zeiss LSM 880 inverted fluorescence microscope (Carl Zeiss). OppA was analyzed before and after encapsulation into liposomes using two-photon excitation of AF555 at 810 nm (80 MHz repetition rate). Samples were deposited on uncoated #1.5 glass coverslips (Marienfeld) and excited through a 40× water immersion objective (Plan

Apochromat, Carl Zeiss). Fluorescence was recorded for 60 s (free OppA) or 120 s (liposome encapsulated OppA).

Emission was collected through the same objective and passed sequentially through a 720+ nm excitation laser blocking filter (Carl Zeiss), a 560 nm long-pass filter, and a 590/50 nm band-pass filter (Becker & Hickl). Photons were detected with an HPM-100-40 single-photon hybrid detector (Becker & Hickl) connected to an SPC-180NX TCSPC module (Becker & Hickl). Photon arrival times were recorded over 12.5 ns acquisition window corresponding to the laser repetition period and binned into 1,024 timing channels.

Data acquisition and analysis were performed using SPCM v9.91 and SPCImage NG v9.0 software (Becker & Hickl), respectively. Fluorescence decay curves were fitted with biexponential decay models using an automatically generated instrument response function.

### **Ensemble fluorescence analysis**

Binding of OppA to RDMPIQAF peptide was monitored by steady-state fluorescence spectroscopy in a Cary Eclipse scanning fluorimeter (Varian) with excitation/emission slit widths set to 5 and 10 nm, respectively. Measurements were performed at room temperature in liposome buffer (25 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 5% (v/v) glycerol). Unlabeled OppA (150 nM) was excited at 280 nm and intrinsic tryptophan fluorescence emission spectra were recorded during titration with RDMPIQAF peptide (0.1–10  $\mu$ M) and under saturating conditions (50  $\mu$ M).

### **LC-MS analysis**

Labeling efficiency, identity and purity of gp100K<sup>AF647</sup> used for refolding of pMHC<sup>AF647</sup> were verified by LC-MS using a BioAccord system (Waters) controlled with UNIFY3.1.0 software. The labeled peptide was separated on an Acquity BEH C4 column (300 Å, 1.7  $\mu$ m, 2.1  $\times$  50 mm, Waters) at 45 °C using a linear acetonitrile gradient (2–80% in 6.5 min) supplemented with 0.1% (v/v) formic acid. Mass spectra were acquired in positive ion mode with a cone voltage of 30 V and a capillary voltage of 0.8 kV.

## **Functionalization of glass coverslides for single-molecule FRET analysis**

Glass coverslides were functionalized via aminosilanization as previously described<sup>71</sup>. Coverslides were cleaned by sequential sonication in Milli-Q water and analytical-grade acetone (>99.9%), followed by oxygen plasma treatment at 0.3 mbar and 80% power for 15 min, and 10 min incubation in methanol. Subsequently, coverslides were incubated for 20 min in silanization solution (100 mL methanol, 5 mL acetic acid, and 3 mL 3-aminopropyltrimethoxysilane). After sonication for 1 min, slides were incubated in silanization solution for 10 min, rinsed with methanol, and dried under a stream of nitrogen gas.

For polyethylene glycol (PEG) functionalization, amino groups were coupled to a mixture of 4% (v/v) biotinylated PEG and 96% (v/v) non-biotinylated PEG via NHS chemistry. For this, 3.5 mg of biotin-PEG-NHS (5,000 Da) were dissolved in 750  $\mu$ L of 0.1 M NaHCO<sub>3</sub> pH 8.5 and 85 mg CH<sub>3</sub>O-PEG-NH-CO-C<sub>2</sub>H<sub>4</sub>-CONHS (5,000 Da) was added. Air bubbles were removed by centrifugation (16,000  $\times$  g, 1 min). The PEG solution was applied between two glass slides and incubated overnight in a humidity chamber.

Coverslides were subsequently rinsed thoroughly with Milli-Q water, dried with nitrogen gas, and subjected to a second PEGylation step using CH<sub>3</sub>-PEG-NHS (333 Da, 25 mM final concentration) dissolved in 0.1 M NaHCO<sub>3</sub> pH 8.5. Following overnight incubation in a humidity chamber, coverslides were rinsed with Milli-Q water, dried with nitrogen gas, and stored at -20 °C under argon until use.

## **Single-molecule FRET imaging**

Single-molecule experiments were conducted as previously described<sup>37</sup> with the modifications outlined below. Flow chambers were assembled by placing a biotin-PEG-functionalized glass slide onto a sticky-slide flow channel (I Luer 0.8 mm, Ibidi), with the functionalized surface facing inward. Flow chambers were connected to a 20 mL syringe via silicon tubing and flushed with filtered liposome buffer (25 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, and 5% (v/v) glycerol).

The biotinylated surface was functionalized with streptavidin (0.2 mg/mL) for 30 min at 4 °C, followed by washing with liposome buffer. Proteoliposomes (5 mg/mL) were clarified by centrifugation (2,000  $\times$  g, 2 min, 4 °C) and immobilized on the surface by incubation for 30 min at 4 °C, followed by washing with liposome buffer.

Remaining streptavidin binding sites were blocked by incubation with biotin (2 mM) for 10 min at 4 °C, followed by washing with liposome buffer. In experiments involving Lck, flow chambers were incubated with  $\alpha$ -hemolysin (2  $\mu$ M) for 45 min at 4 °C, followed by washing with liposome buffer. The chambers were finally washed five times with liposome buffer and exchanged to imaging buffer (25 mM HEPES-NaOH pH 7.2, 150 mM NaCl, 5% (v/v) glycerol, 90 mM glucose, 5 mM Trolox, 7.5 U/mL pyranose oxidase, 1 kU/mL catalase).

Single-molecule measurements were performed using alternating laser excitation (ALEX) on a total internal reflection fluorescence (TIRF) microscope maintained at 30 °C (Nanoimager S, Oxford Nanoimaging). Typically, 600 frames were acquired per ROI using an exposure time of 100 ms. Excitation laser powers were set to 0.8 mW/cm<sup>2</sup> (532 nm) and 0.9 mW/cm<sup>2</sup> (640 nm). Data were recorded in 1-min intervals over 80 min before pMHC ligands (100 nM final concentration) or MgCl<sub>2</sub> and ATP (both 10 mM final concentration) were added to immobilized liposomes, and acquisition was continued. For photobleaching experiments, fluorophores were continuously illuminated at 640 nm (0.9 mW/cm<sup>2</sup>), and bleaching events were recorded.

### Single-molecule FRET data analysis

Single-molecule FRET data were analyzed as previously described<sup>37</sup>. ALEX was used to separate detected photons based on excitation and emission channels. Photons were assigned to three fluorescence classes: donor excitation with donor emission ( $F_{DD}$ ), donor excitation with acceptor emission ( $F_{DA}$ ), and acceptor excitation with acceptor emission ( $F_{AA}$ ). Using the Nanoimager software (ONI Nanoimager Development Build), FRET efficiency ( $E$ ) and stoichiometry ( $S$ ) values were calculated, and FRET trajectories were extracted for subsequent analysis in deepFRET (ref.<sup>72</sup>). Automated trace selection was performed using an 80% confidence threshold, followed by manual curation to ensure data quality. FRET efficiency distributions were generated from selected trajectories and fitted using Gaussian mixture modeling to determine the relative populations of low- and high-FRET states. Photobleaching experiments were analyzed by Deep-LASI (ref.<sup>73</sup>).

## **Graph design and statistical analysis**

Figures displaying cryo-EM densities or structural models were prepared using UCSF ChimeraX (ref.<sup>68</sup>). Receptors were aligned to the membrane plane using membrane coordinates (PDB: 7PHR) from the OPM database (ref.<sup>74</sup>). For visualization of lipid densities, cryo-EM maps were subjected to local sharpening in cryoSPARC. Cryo-EM workflow data were generated in cryoSPARC. Chemical structures were drawn in ChemDraw. Representative fluorescence micrographs were created with the FiJi distribution of ImageJ (ref.<sup>75</sup>) by averaging 10 consecutive frames. Here, the background was corrected by subtraction of empty reference micrographs (smFRET data) or the averaged final 50 frames of each movie (photobleaching data). Other data were analyzed and plotted in OriginPro (OriginLabs).

Quantitative data are presented as mean  $\pm$  standard deviation (SD) unless otherwise indicated. The number of independent experiments and analyzed molecules, particles, or technical replicates are specified in the corresponding figure legends. No data points were excluded unless explicitly stated. Quantification and analysis details are described in each relevant section of the methods or figure legends.

## **Data reporting**

No statistical methods were used to determine sample size. The experiments were not randomized, and the investigators were not blinded to allocation during the experiments or to the outcome assessment.

## **Reporting summary**

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

## **Data availability**

All data supporting the findings of this study are available within the main text, the Supplementary Information, and the accompanying Source Data file (DOI: <https://doi.org/10.25716/gude.0rwv-tvxg>). Additional data are available from the corresponding authors upon reasonable request.

Atomic coordinates have been deposited in the Protein Data Bank (PDB) under accession numbers 9U0H (pMHC/TCR<sup>GPa3b17</sup> in MSP2N2), 9U0J (TCR<sup>GPa3b17</sup> in MSP2N2), 9U0K (TCR<sup>GPabWT</sup> in MSP2N2), 9U0L (pMHC/TCR<sup>GPa3b17</sup> in GDN), 9U0M (TCR<sup>GPa3b17</sup> in GDN), and 9U0N (TCR<sup>GPabWT</sup> in GDN) (see [Key Resources Table](#)).

Cryo-EM maps have been deposited in the Electron Microscopy Data Bank (EMDB) under accession numbers EMD-56491 (pMHC/TCR<sup>GPa3b17</sup> in MSP2N2), EMD-56493 (TCR<sup>GPa3b17</sup> in MSP2N2), EMD-56494 (TCR<sup>GPabWT</sup> in MSP2N2), EMD-56495 (pMHC/TCR<sup>GPa3b17</sup> in GDN), EMD-56496 (TCR<sup>GPa3b17</sup> in GDN), and EMD-56497 (TCR<sup>GPabWT</sup> in GDN) (see [Key Resources Table](#)).

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## Author contributions

M.Z. performed production, purification, reconstitution, and biochemical analyses of TCR complexes. F.O. and L.S. carried out cryo-EM sample preparation, data collection, and single particle analyses. M.Z. and Y.B. built and refined structural models. C.N. prepared OppA, functionalized glass coverslips, and performed single-molecule imaging and analyses. D.G. synthesized peptides and performed TCSPC measurements. M.D. prepared TCR complexes in MSP1D1 nanodiscs. M.Z., L.S. and R.T. wrote the manuscript with contributions from all authors. R.T. conceived and supervised the study.

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## Competing interests

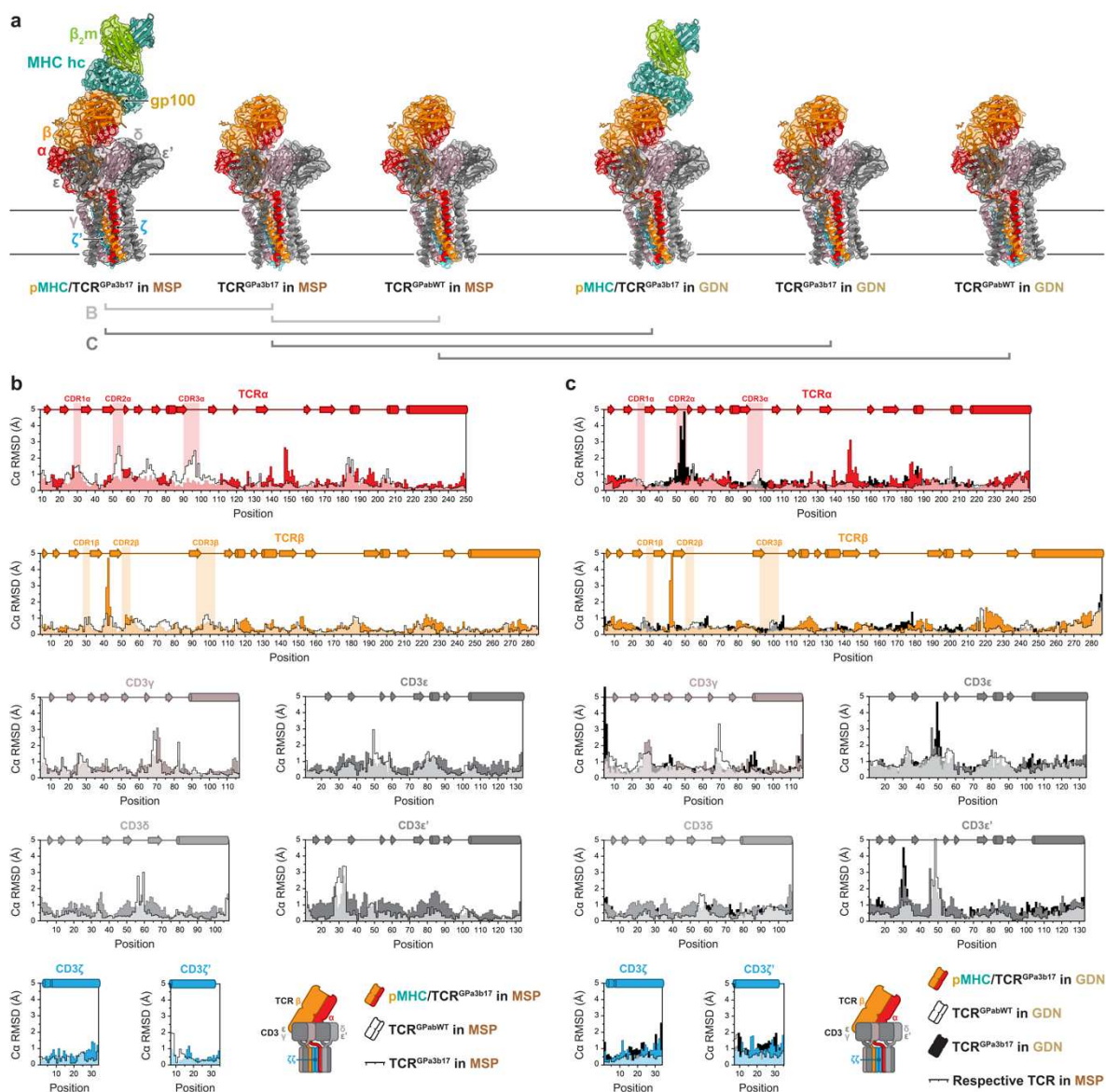
The authors declare no competing interests.

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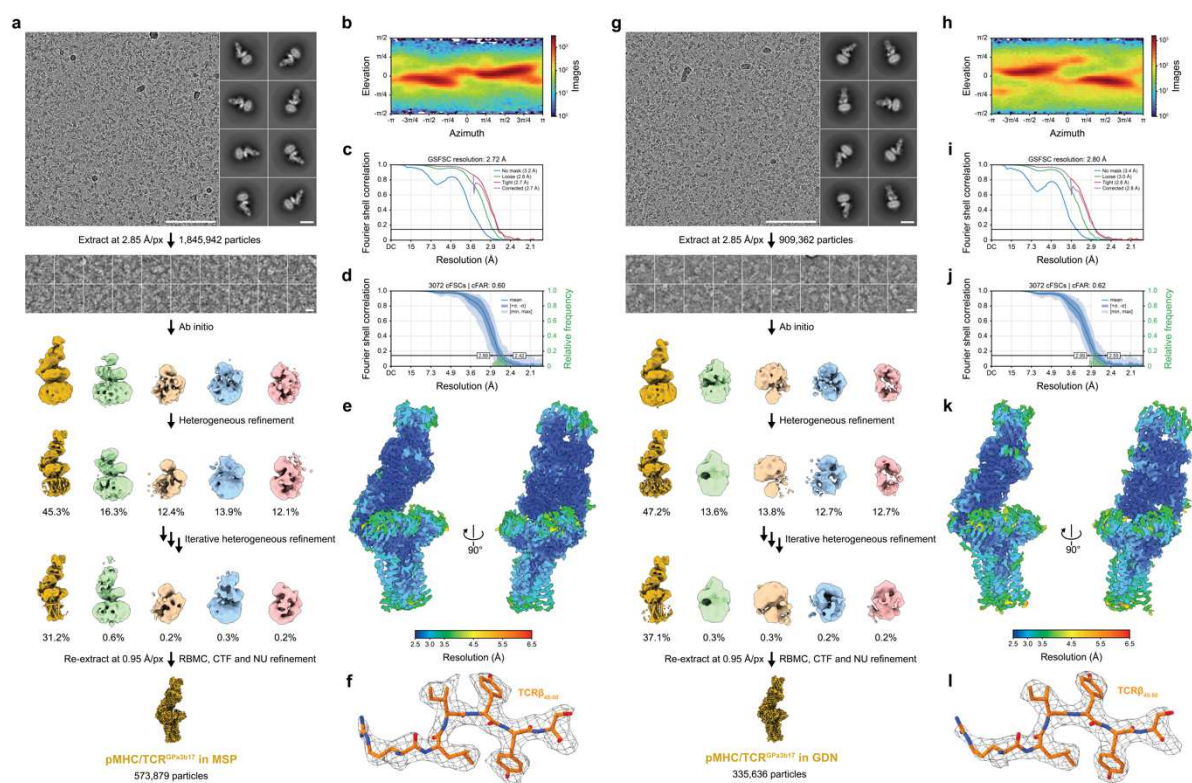
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## Extended Data Figures



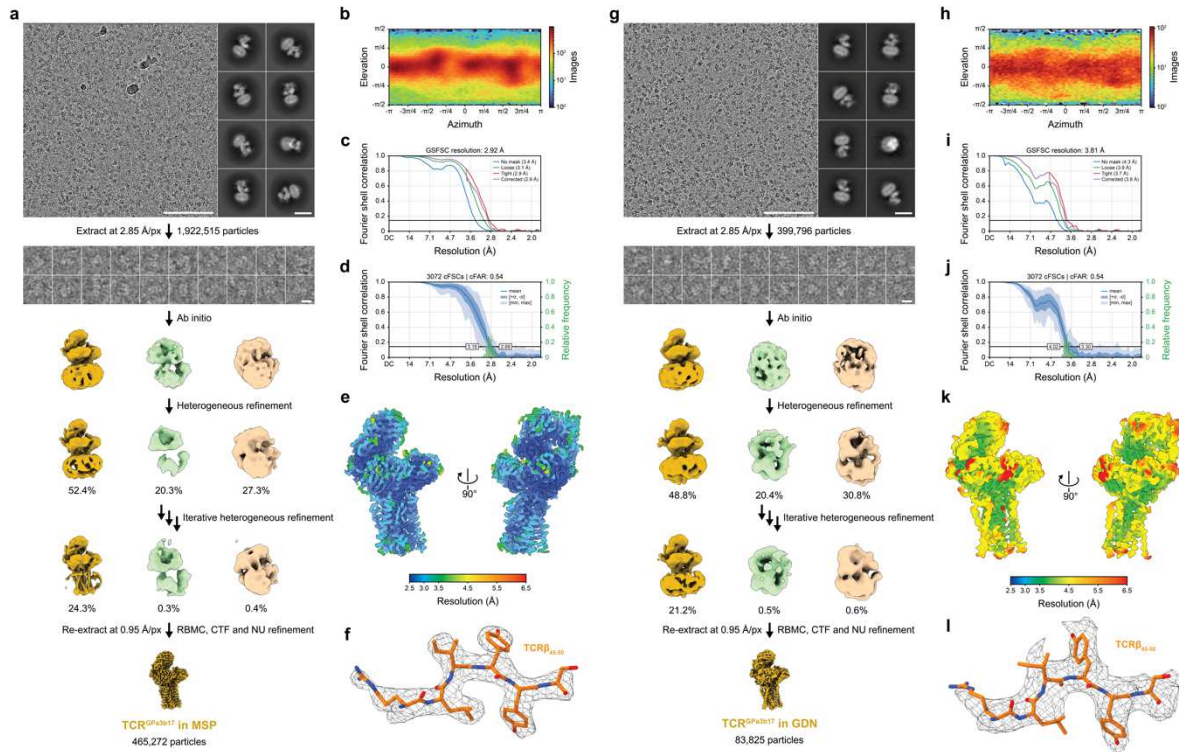
**Extended Data Fig. 1 | Structural comparisons demonstrate conservation of the TCR architecture across ligand, affinity, and membrane conditions.** **a**, Overview of the six TCR structures determined in this study and schematic summary of the structural comparisons presented in panels (b) and (c). **b**, Ca root-mean-square deviations (RMSDs) plotted as a function of residue position for each TCR subunit. The apo affinity-enhanced TCR<sup>GPa3b17</sup> structure serves as a reference. Comparisons with pMHC-bound TCR<sup>GPa3b17</sup> are shown in color, whereas comparisons with the parental TCR<sup>GPabWT</sup> are shown as semi-transparent white bars. Secondary structure elements are indicated above each plot. Shaded regions denote the complementarity-determining regions (CDR1–3) of TCR $\alpha$  and TCR $\beta$ , which contain the affinity-enhancing substitutions (see Fig. 2). **c**, Ca RMSDs plotted as function of residue

position comparing TCR complexes reconstituted in MSP2N2 lipid nanodiscs (MSP) and GDN detergent micelles. Three comparisons are shown for such subunit: pMHC-bound TCR<sup>GPa3b17</sup> (colored), apo TCR<sup>GPa3b17</sup> (black) and apo parental TCR<sup>GPa<sup>b</sup>WT</sup> (white). Secondary structure elements are indicated above each plot.

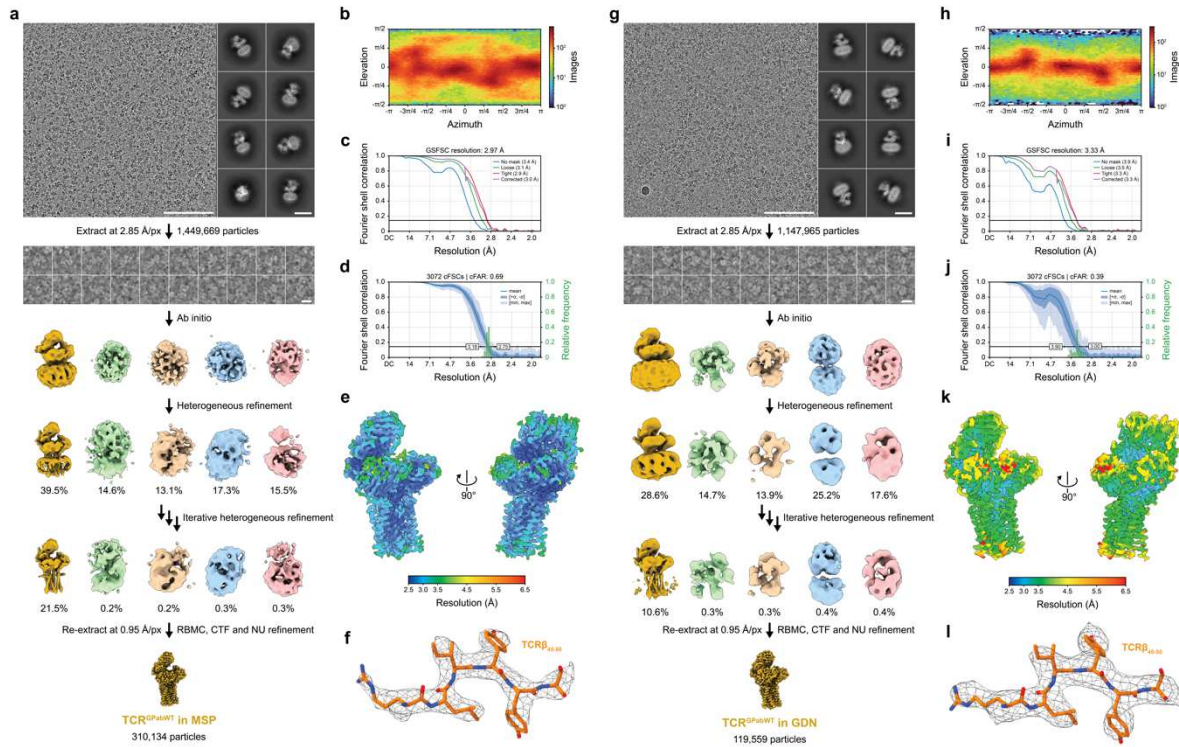


**Extended Data Fig. 2 | Cryo-EM data processing and validation of the ligand-bound affinity-enhanced pMHC/TCR<sup>GPa3b17</sup> complex in lipid nanodiscs and detergent micelles.**

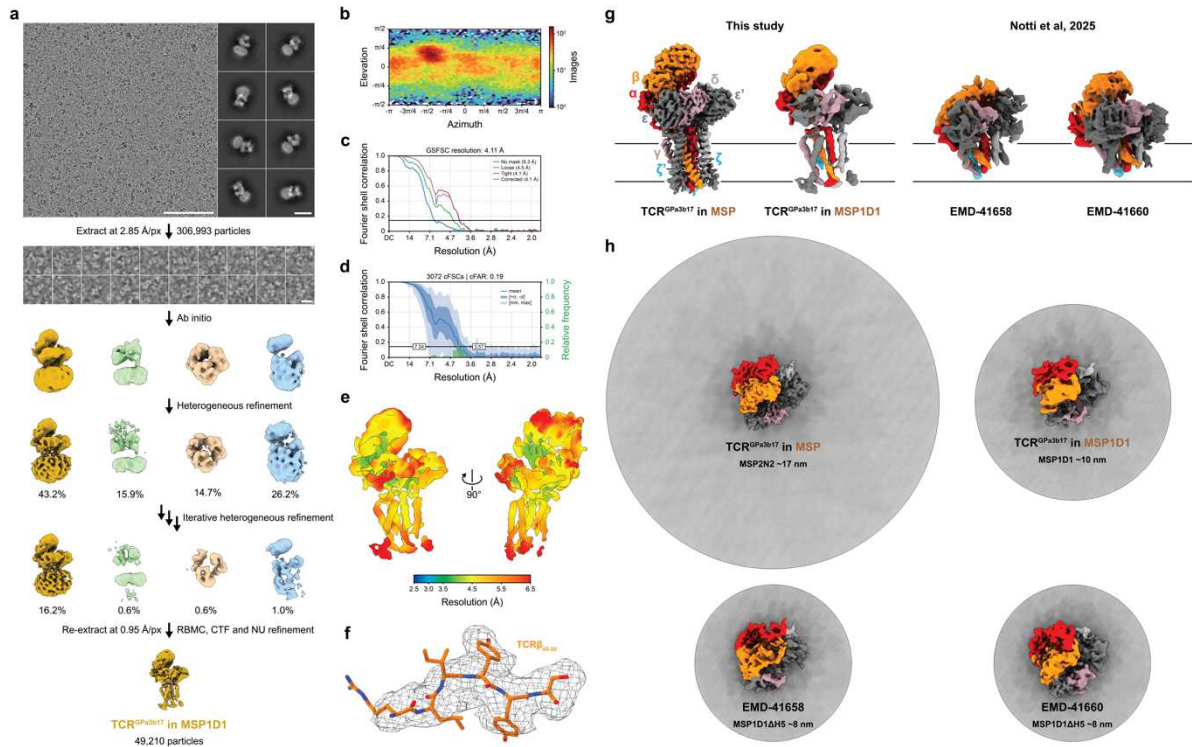
**a,g**, Cryo-EM data processing scheme for the ligand-bound affinity-enhanced pMHC–TCR<sup>GPa3b17</sup> complex reconstituted in MSP2N2 lipid nanodiscs (left; **a–f**) and GDN detergent micelles (right; **g–l**). Representative micrograph (scale bar, 100 nm), selected 2D class averages (scale bar, 10 nm), 3D classification and refinement strategy, and the final cryo-EM density map are shown. Absolute particle numbers are indicated for the initial and final datasets, whereas relative particle numbers are shown for intermediate processing steps. **b,h**, Angular distribution of particles contribution to the final reconstruction. **c,i**, Gold-standard Fourier shell correlation (GSFSC) curve of the final reconstruction. The horizontal dashed line indicates the FSC = 0.143 criterion used to estimate the overall map resolution. **d,j**, Conical Fourier shell correlation (cFSC) analysis and corresponding cFSC anisotropy ratio (cFAR) assessing directional resolution. **e,k**, Final cryo-EM density map colored according to local resolution. **f,l**, Representative cryo-EM density for TCRβ residues 45–50 (RLIYYS), illustrating the quality of the reconstructed density and the level of structural detail achieved throughout the TCR complex.



**Extended Data Fig. 3 | Cryo-EM data processing and validation of the apo affinity-enhanced TCR<sup>GPa3b17</sup> complex in lipid nanodiscs and detergent micelles.** **a,g**, Cryo-EM data processing scheme for the apo affinity-enhanced TCR<sup>GPa3b17</sup> complex reconstituted in MSP2N2 lipid nanodiscs (left; **a–f**) and GDN detergent micelles (right; **g–l**). Representative micrograph (scale bar, 100 nm), selected 2D class averages (scale bar, 10 nm), 3D classification and refinement strategy, and the final cryo-EM density map are shown. Absolute particle numbers are indicated for the initial and final datasets, whereas relative particle numbers are shown for intermediate processing steps. **b,h**, Angular distribution of particles contribution to the final reconstruction. **c,i**, Gold-standard Fourier shell correlation (GSFSC) curve of the final reconstruction. The horizontal dashed line indicates the FSC = 0.143 criterion used to estimate the overall map resolution. **d,j**, Conical Fourier shell correlation (cFSC) analysis and corresponding cFSC anisotropy ratio (cFAR) assessing directional resolution. **e,k**, Final cryo-EM density map colored according to local resolution. **f,l**, Representative cryo-EM density for TCRβ residues 45–50 (RLIYYS), illustrating the quality of the reconstructed density and the level of structural detail achieved throughout the TCR complex.

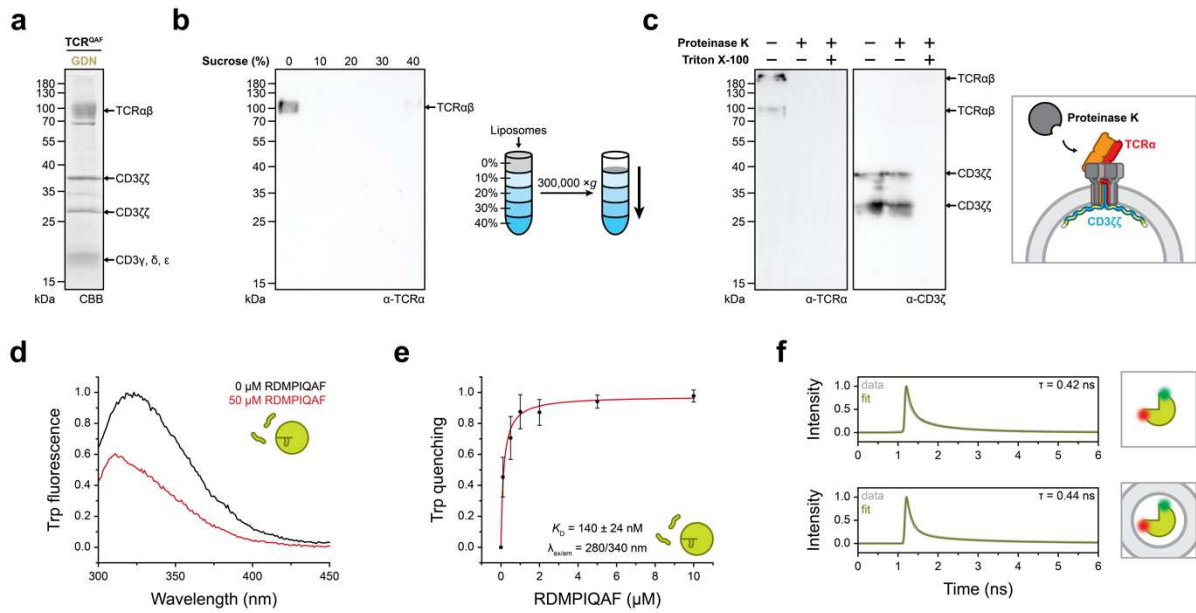


**Extended Data Fig. 4 | Cryo-EM data processing and validation of the apo parental TCR<sup>GPa3b17</sup> complex in lipid nanodiscs and detergent micelles.** **a,g**, Cryo-EM data processing scheme for the apo parental TCR<sup>GPa3b17</sup> complex reconstituted in MSP2N2 lipid nanodiscs (left; **a–f**) and GDN detergent micelles (right; **g–l**). Representative micrograph (scale bar, 100 nm), selected 2D class averages (scale bar, 10 nm), 3D classification and refinement strategy, and the final cryo-EM density map are shown. Absolute particle numbers are indicated for the initial and final datasets, whereas relative particle numbers are shown for intermediate processing steps. **b,h**, Angular distribution of particles contribution to the final reconstruction. **c,i**, Gold-standard Fourier shell correlation (GSFSC) curve of the final reconstruction. The horizontal dashed line indicates the FSC = 0.143 criterion used to estimate the overall map resolution. **d,j**, Conical Fourier shell correlation (cFSC) analysis and corresponding cFSC anisotropy ratio (cFAR) assessing directional resolution. **e,k**, Final cryo-EM density map colored according to local resolution. **f,l**, Representative cryo-EM density for TCRβ residues 45–50 (RLIYYs), illustrating the quality of the reconstructed density and the level of structural detail achieved throughout the TCR complex.

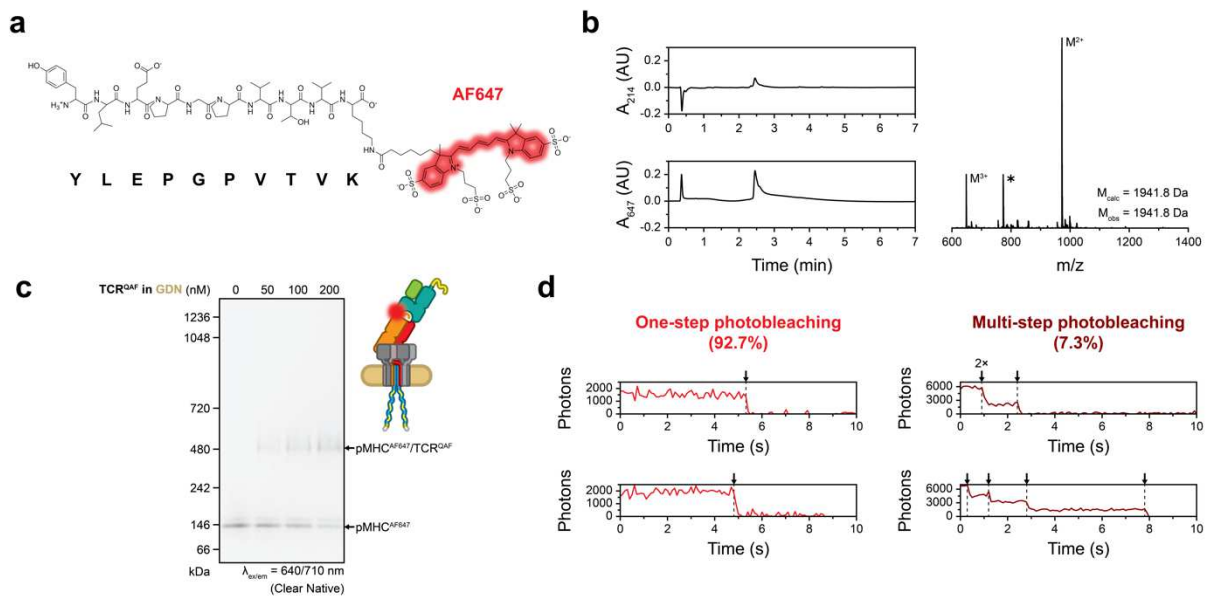


**Extended Data Fig. 5 | Membrane confinement by small nanodiscs compromises TCR structural integrity.** **a**, Cryo-EM data processing scheme for apo affinity-enhanced TCR<sup>GPa3b17</sup> complex reconstituted in MSP1D1 lipid nanodiscs. Representative micrograph (scale bar, 100 nm), selected 2D class averages (scale bar, 10 nm), 3D classification and refinement strategy, and the final cryo-EM density map are shown. Absolute particle numbers are indicated for the initial and final datasets, whereas relative particle numbers are shown for intermediate processing steps. **b**, Angular distribution of particles contribution to the final reconstruction. **c**, Gold-standard Fourier shell correlation (GSFSC) curve of the final reconstruction. The dashed horizontal line indicates the FSC = 0.143 criterion used to estimate global resolution. **d**, Conical Fourier shell correlation (cFSC) analysis and corresponding cFSC anisotropy ratio (cFAR) of the final reconstruction. **e**, Final cryo-EM density map colored according to local resolution. **f**, Representative cryo-EM density for TCRβ residues 45–50 (RLIYYS), illustrating local map quality. The atomic model of TCR<sup>GPa3b17</sup> reconstituted in MSP2N2 was rigid-body fitted into the TCR<sup>GPa3b17</sup> in MSP1D1 reconstruction for visualization. **g**, Side-view comparison of TCR cryo-EM maps obtained in nanodiscs of varying diameters. Maps are color-coded by subunit identity. Reconstructions from small nanodiscs are poorly resolved, particularly within the cytosolic half of the transmembrane assembly, whereas large MSP2N2 nanodiscs preserve a continuous and well-resolved receptor architecture. **h**, Top-view comparison of the reconstructions shown in (G). Grey circles indicate

the approximate nanodisc diameter. Larger nanodiscs provide substantially improved structural integrity and isotropic reconstruction of the TCR complex.



**Extended Data Fig. 6 | Validation of TCR<sup>QAF</sup> reconstitution and smFRET assay components.** **a**, Purity and integrity of purified TCR<sup>QAF</sup> complexes assessed by Coomassie-stained non-reducing SDS-PAGE. The full-length CD3ζζ homodimer displays an atypical migration pattern due to its intrinsically disordered cytosolic regions. **b**, Sucrose density gradient analysis of TCR<sup>QAF</sup>-containing proteoliposomes. Non-reducing immunoblotting demonstrates that TCR complexes partition with the liposome-containing fraction, confirming efficient incorporation and excluding substantial receptor aggregation. **c**, Protease protection analysis verifies the physiological orientation of reconstituted TCR<sup>QAF</sup> complexes. Selective degradation of the extracellular TCRαβ domains and protection of the cytosolic CD3ζζ domains demonstrate ectodomain-outward insertion. Aggregated TCRαβ species resulting from TCA precipitation are visible by non-reducing immunoblot. **d,e**, Binding of OppA to soluble RDMPIQAF peptide was characterized by tryptophan quenching experiments. Conformational changes in OppA increased solvent access of tryptophan residues, quenching fluorescence (mean  $\pm$  SD,  $n = 3$  independent experiments). **f**, TCSPC measurements of OppA fluorescence lifetime before and after encapsulation into liposomes. Comparable lifetimes indicate that membrane encapsulation does not alter OppA photophysical properties.



**Extended Data Fig. 7 | Production, labeling and characterization of pMHC<sup>AF647</sup> for photobleaching experiments.** **a**, Chemical structure of gp100K<sup>AF647</sup> used for refolding of pMHC<sup>AF647</sup> to obtain pMHC labeled with exactly one fluorophore. **b**, Quality control of gp100K<sup>AF647</sup> peptide used for refolding of pMHC<sup>AF647</sup>. LC–MS analysis confirms peptide identity and purity. Chromatograms (left) demonstrate sample homogeneity, mass spectra (right) confirm the expected product species. The asterisk denotes a common AF647 fragment<sup>76</sup>. **c**, Fluorophore labeling of pMHC<sup>AF647</sup> does not impair TCR<sup>QAF</sup> binding at concentrations used for photobleaching experiments, validated by Clear Native PAGE and subsequent in-gel fluorescence. **d**, Representative photobleaching traces for pMHC<sup>AF647</sup>, showing one-step (left) and multi-step (right) bleaching behavior. Single-molecule bleaching events are indicated (arrow and dotted line).

## Extended Data Tables

**Extended Data Table 1. Cryo-EM structure determination and validation statistics**

| Structure                                       | pMHC/TCR <sup>GPa3b17</sup><br>in MSP | TCR <sup>GPa3b17</sup><br>in MSP | TCR <sup>GPa3bWT</sup><br>in MSP | pMHC/TCR <sup>GPa3b17</sup><br>in GDN | TCR <sup>GPa3b1</sup><br>in GDN | TCR <sup>GPa3bWT</sup><br>in GDN |
|-------------------------------------------------|---------------------------------------|----------------------------------|----------------------------------|---------------------------------------|---------------------------------|----------------------------------|
| <b>Cryo-EM sample conc.</b>                     | 5.5 mg/mL<br>(19 $\mu$ M)             | 3.3 mg/mL<br>(14 $\mu$ M)        | 3.6 mg/mL<br>(15 $\mu$ M)        | 3.2 mg/mL<br>(16 $\mu$ M)             | 2.7 mg/mL<br>(18 $\mu$ M)       | 3.4 mg/mL<br>(22 $\mu$ M)        |
| <b>Data collection</b>                          |                                       |                                  |                                  |                                       |                                 |                                  |
| <b>Movies</b>                                   | 11,673                                | 13,758                           | 9,252                            | 9,748                                 | 12,071                          | 9,686                            |
| <b>Initial particles</b>                        | 1,845,942                             | 1,922,515                        | 1,449,669                        | 909,362                               | 399,796                         | 1,147,965                        |
| <b>Final particles</b>                          | 573,879                               | 465,272                          | 310,134                          | 335,636                               | 83,825                          | 119,559                          |
| <b>GSFSC resolution (Å)</b>                     | 2.72                                  | 2.92                             | 2.97                             | 2.80                                  | 3.81                            | 3.33                             |
| <b>Model composition</b>                        |                                       |                                  |                                  |                                       |                                 |                                  |
| <b>Non-H atoms</b>                              | 11,697                                | 8,544                            | 8,522                            | 11,725                                | 8,530                           | 8,527                            |
| <b>Amino acid residues</b>                      | 1,448                                 | 1,064                            | 1,062                            | 1,448                                 | 1,058                           | 1,059                            |
| <b>Ligands</b>                                  | 10 NAG                                | 10 NAG                           | 10 NAG                           | 10 NAG, 1 CLR                         | 10 NAG, 1 CLR                   | N10 NAG, 1 CLR                   |
| <b>Bond R. m. s. deviations</b>                 |                                       |                                  |                                  |                                       |                                 |                                  |
| <b>Length (Å)</b>                               | 0.004                                 | 0.004                            | 0.003                            | 0.003                                 | 0.004                           | 0.003                            |
| <b>Angles (°)</b>                               | 0.540                                 | 0.523                            | 0.498                            | 0.505                                 | 0.627                           | 0.479                            |
| <b>Validation</b>                               |                                       |                                  |                                  |                                       |                                 |                                  |
| <b>MolProbity score</b>                         | 1.39                                  | 1.29                             | 1.13                             | 1.13                                  | 1.29                            | 1.15                             |
| <b>Clash score</b>                              | 4.3                                   | 3.55                             | 2.61                             | 3.03                                  | 5.44                            | 3.37                             |
| <b>Rotamer outliers (%)</b>                     | 0.00                                  | 0.00                             | 0.00                             | 0.00                                  | 0.00                            | 0.00                             |
| <b>C<math>\beta</math> outliers (%)</b>         | 0.07                                  | 0.10                             | 0.00                             | 0.07                                  | 0.10                            | 0.00                             |
| <b>Ramachandran plot (%)</b>                    |                                       |                                  |                                  |                                       |                                 |                                  |
| <b>Outliers</b>                                 | 0.00                                  | 0.00                             | 0.00                             | 0.00                                  | 0.00                            | 0.00                             |
| <b>Allowed</b>                                  | 3.09                                  | 2.77                             | 2.39                             | 2.17                                  | 1.73                            | 2.11                             |
| <b>Favored</b>                                  | 96.91                                 | 97.23                            | 97.61                            | 97.83                                 | 98.27                           | 97.89                            |
| <b>Rama-Z (Ramachandran plot Z-score, RMSD)</b> |                                       |                                  |                                  |                                       |                                 |                                  |
| <b>Whole</b>                                    | 0.11 (0.22)                           | -0.58 (0.26)                     | -0.11 (0.26)                     | -0.25 (0.22)                          | 0.20 (0.25)                     | 0.47 (0.26)                      |

|                                                |                    |                     |                    |                    |                     |                     |
|------------------------------------------------|--------------------|---------------------|--------------------|--------------------|---------------------|---------------------|
| <b>Helix</b>                                   | 1.84 (0.27)        | -0.01 (0.31)        | 0.81 (0.32)        | -0.23 (0.28)       | 0.87 (0.31)         | 1.52 (0.30)         |
| <b>Sheet</b>                                   | -0.65 (0.25)       | -0.21 (0.31)        | -0.43 (0.33)       | 0.21 (0.26)        | 0.45 (0.34)         | 0.11 (0.35)         |
| <b>Loop</b>                                    | -0.36 (0.24)       | -0.58 (0.28)        | -0.23 (0.27)       | -0.24 (0.24)       | -0.30 (0.25)        | -0.14 (0.27)        |
| <b>B factors, min/max/mean (Å<sup>2</sup>)</b> |                    |                     |                    |                    |                     |                     |
| <b>Protein</b>                                 | 5.25/191.63/63.08  | 7.26/136.73/61.48   | 20.74/119.35/64.07 | 19.17/166.11/60.44 | 21.81/160.37/91.00  | 15.24/167.02/82.72  |
| <b>Ligand</b>                                  | 64.32/128.41/98.23 | 64.67/139.83/101.35 | 66.28/111.03/85.96 | 62.58/106.80/78.95 | 81.26/166.42/113.57 | 63.98/156.54/113.56 |
| <b>Model vs. Data</b>                          |                    |                     |                    |                    |                     |                     |
| <b>CC (mask)</b>                               | 0.81               | 0.84                | 0.82               | 0.83               | 0.75                | 0.75                |
| <b>CC (box)</b>                                | 0.76               | 0.75                | 0.72               | 0.74               | 0.63                | 0.59                |
| <b>CC (peaks)</b>                              | 0.72               | 0.71                | 0.68               | 0.70               | 0.55                | 0.55                |
| <b>CC (volume)</b>                             | 0.81               | 0.83                | 0.81               | 0.82               | 0.74                | 0.73                |
| <b>Mean CC for ligands</b>                     | 0.62               | 0.64                | 0.67               | 0.66               | 0.63                | 0.60                |
| <b>Deposition ID</b>                           |                    |                     |                    |                    |                     |                     |
| <b>PDB</b>                                     | 9U0H               | 9U0J                | 9U0K               | 9U0L               | 9U0M                | 9U0N                |
| <b>EMDB</b>                                    | 56491              | 56493               | 56494              | 56495              | 56496               | 56497               |

## Supplementary Files

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