

# Structural and computational interrogation of an Influenza A Matrix protein-targeting TCR

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## Article

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# Abstract

Structural understanding of T cell receptor (TCR) complexes is fundamental to deciphering immune recognition and signaling, with clear clinical relevance to infectious disease, cancer, and autoimmunity. Here, we present cryo-EM structures of a full-length TCR/CD3 complex—engineered to respond to an influenza peptide—both in the absence and presence of peptide-loaded MHCI ligand. Our results show that ectodomain TCR engineering preserves overall complex assembly and that peptide engagement induces localized conformational changes without altering the global architecture. By integrating lipidomics and computational modeling, we identify novel lipid binding sites within the TCR/CD3 complex, suggesting further potential regulatory roles for membrane lipids in TCR function. Additionally, using molecular dynamics simulations in tandem with surface plasmon resonance, we predict and confirm determinants of TCR affinity against mutant peptides and MHC surface residues. These findings provide new insights into the molecular organization and flexibility of the TCR/CD3 complex, offering a platform for future studies and engineering of T cell signaling and immune modulation.

## Introduction

T-cell receptors (TCRs) play a critical role in adaptive immunity by recognizing antigenic peptides presented by major histocompatibility complex (MHC) molecules, initiating downstream signaling crucial for T-cell activation [1–4]. Engineering of T cell responses has garnered increasing interest alongside the development of therapeutic modalities such as CAR-T and bispecific T-cell engagers (BiTEs), with potential applications against cancer, infectious disease, and autoimmunity [5]. While engineered T-cell-based therapeutics have been applied successfully against cancer [6] and more recently autoimmune diseases [7], the engineering of T-cell responses against infectious disease has seen less progress. In part, several unique considerations such as off-target toxicity and a preference for sequence recognition breadth over specificity [8] may have limited application of these technologies to infectious disease. Given these unique challenges, we hypothesize that a detailed structural understanding of engineered TCR recognition and signaling against viral antigens provides a platform for development of infectious-disease-targeted T-cell-based therapeutics.

The TCR is a heterodimer formed by TCR  $\alpha$  and TCR  $\beta$  chains, in which the variable domains of  $\alpha$  and  $\beta$  form contact with pMHC molecules via complementarity determining regions (CDRs) [9]. TCR does not in itself contain functional intracellular signaling domains; rather, cluster of differentiation 3 protein (CD3) is noncovalently complexed with TCR and CD3 plays a key role in T cell signaling via its Immunoreceptor Tyrosine-Based Activation Motifs (ITAMs). T cell signaling is initiated by phosphorylation of ITAMs which is followed by multiple downstream signaling cascade events [10]. Thus, structural insights into the full-length TCR/CD3 complex are instrumental to understanding the mechanisms of antigen recognition, signal transduction, and immune response modulation. Early studies have shown that the TCR/CD3 complex comprises one heterodimer of TCR  $\alpha$  and  $\beta$  chains, one heterodimer of CD3 $\delta\epsilon$ , one heterodimer of CD3 $\gamma\epsilon$  and one homodimer of CD3 $\zeta\zeta$  [11]. More recent studies have elucidated structures of full-length TCR/CD3 complexes in different immune contexts,

including some derived from healthy donors and some that were essential in cancer-related immune responses [12–15]. However, the structural basis of virus-derived antigen pMHC recognition within the full-length TCR/CD3 framework remains largely unexplored.

To address this gap, we investigated a full-length TCR/CD3 complex engineered to recognize the influenza A matrix protein derived M1(58–66) peptide presented by the HLA-A\*02:01 allele in pMHC complexes. The M1(58–66) epitope (58-GILGFVFTL-66) is well-characterized and important in T-cell activity to clear influenza A infections, and the soluble JM22 TCR<sup>M1(58–66)</sup>/pMHC<sup>M1(58–66)</sup> complex has been structurally characterized [16, 17]. By transplanting the ectodomains of this TCR onto the constant domains of a previously structurally characterized full-length TCR/CD3 complex (PDB 6JXR), we aimed to assess whether such domain substitution would alter the assembly, stability, or spatial configuration of the TCR/CD3 machinery. Moreover, the full-length context enables examination of membrane-proximal features such as glycosylation, transmembrane architecture, and lipid interactions—elements absent in crystallographic studies of truncated constructs.

An emerging dimension in TCR signaling is the role of lipid microenvironments. In particular, the determination of full-length TCR structures in the context of various detergent and nanodisc formulations demonstrates significant conformational differences, which may provide insight into mechanisms of TCR activation and signaling [18]. The plasma membrane is not a passive bilayer but a dynamic landscape composed of cholesterol-rich domains, phosphoinositides, sphingolipids, and glycerophospholipids that modulate receptor clustering and activation. Specifically, phosphoinositides—particularly PI(4,5)P<sub>2</sub>—have been implicated in promoting localized signaling by recruiting adaptor proteins and supporting CD3 ITAM exposure through interactions at the cytoplasmic face of the membrane [19–23]. Despite these functional insights, high-resolution structural data capturing lipid-TCR interactions remain sparse. Previously reported TCR/CD3 cryo-EM density maps include unresolved or ambiguous lipid-like densities near the membrane, often attributed to detergent artifacts or incompletely characterized native lipids [12, 15]. By combining experimental lipidomics with molecular dynamics simulations using LipIDens [24], our study sought to resolve the identity and potential functional significance of lipid species associated with the TCR/CD3 complex. Specifically, we aimed to test whether these lipid-binding sites are structurally conserved or exhibit preferential lipid occupancy that might relate to TCR activation states or membrane curvature adaptation.

An additional dimension of TCR recognition of viral antigens concerns the determinants of affinity between a TCR and its peptide-MHC target. Although each TCR/pMHC complex may have unique features contributing to affinity and recognition, we hypothesize that core principles such as hydrogen-bond stability and peptide conformational variability lend explanatory power to our understanding of the recognition complex. Using established simulation methods, we are able to use hydrogen bond stability measurements to generate affinity predictions for mutations to the TCR in correspondence with previously collected experimental affinity data. Additionally, we employ our simulation methodology to predict how peptide mutations impact TCR affinity and we validate our predictions by means of SPR experiments.

Overall, our study provides a detailed structural framework for understanding how a viral peptide-loaded pMHC I is recognized by a full-length TCR/CD3 complex, providing additional insights into the molecular mechanisms underlying TCR assembly, antigen engagement, and lipid-mediated modulation. Our findings have implications for future development of TCR-based immunotherapies against infectious disease in particular, with insights applicable to therapeutic design for autoimmunity, cancer, and other diseases as well.

## Results

### Cryo-EM structure of apo-TCR<sup>M1(58–66)</sup>/CD3 complex

Although previous studies have revealed several full-length TCR/CD3 complex structures [12–15], these were either isolated from healthy donors or contained cancer-related TCRs. We sought to understand TCR recognition of a viral antigen peptide-loaded pMHC I in the full-length TCR/CD3 context and to understand whether ectodomain-swap TCR designs would affect TCR/CD3 assembly. We swapped the ectodomains of TCR  $\alpha$  and  $\beta$  chains from the published full length TCR/CD3 complex (6JXR) with those of the published crystal structure of the JM22 TCR<sup>M1(58–66)</sup> (PDB 2VLJ) which recognizes the influenza A virus matrix M1(58–66) peptide presented by HLA-A\*02:01 pMHC I complexes [17]. We designed DNA expression plasmids for TCR<sup>M1(58–66)</sup>/CD3 expression in mammalian suspension cell culture. Here, TCR  $\alpha$  and  $\beta$  chains were separated by the E2A self-cleavage peptide on our TCR expression plasmid whereas CD3  $\epsilon$ ,  $\gamma$ ,  $\delta$  and  $\zeta$  chains were separated by T2A, P2A and E2A peptides, respectively, on our CD3 expression plasmid. We expressed the full length TCR<sup>M1(58–66)</sup>/CD3 complex in Expi293 cells by co-transfecting TCR and CD3 plasmids. Purified complexes were stabilized in detergent as previously described [15]. We determined a cryo-EM structure of apo-TCR<sup>M1(58–66)</sup>/CD3 at a resolution of 3.9 Å (Figs. 1A, S1A and S7). Our density map shows clear density for the side chains on the transmembrane domains and extracellular domains (Figure S1A). N-linked glycosylation sites on TCR  $\alpha$  and  $\beta$  chains were modeled in density (N18, N24, N161, N206 and N195 on TCR  $\alpha$  chain, N18 and N197 on TCR  $\beta$  chain) and they were distinct glycosylation sites from other published TCR/CD3 full length structures. Glycosylation sites on CD3  $\gamma$  and  $\delta$  chains were in identical positions to other published TCR/CD3 full length structures (Fig. 1B). When compared to other published TCR/CD3 complexes, the overall molecular arrangement of TCR<sup>M1(58–66)</sup>/CD3 was similar (Fig. 1C). However, in our single residue root-mean-square-deviation (RMSD) scanning analysis, TCR  $\alpha$  and  $\beta$  chain variable domains (V $\alpha$  and V $\beta$ ) exhibit the larger deviation with an average of 5 Å due to the differences in V $\alpha$  and V $\beta$  sequences between complexes (Figure S1C). Comparing with TCR/CD3 PDB 9BBC [18], each chain had shifted at their transmembrane  $\alpha$ -helix C-terminals rather dramatically (Fig. 1D), but comparing with TCR/CD3 PDBs 7FJF, 7FJD, 7FJE, 8ES7 and 6JXR, TCR  $\alpha$ , TCR  $\beta$  and CD3  $\delta$  exhibited less of a shift (Figs. 1E–1I and S1C). Surprisingly, our TCR<sup>M1(58–66)</sup>/CD3 exhibited a nearly 5 Å shift of either CD3  $\epsilon$  chain transmembrane  $\alpha$ -helix C-termini compared to the parental PDB 6JXR TCR/CD3 structure used in designing our TCR<sup>M1(58–66)</sup>/CD3 constructs. This transmembrane domain positional discrepancy may be

caused by the cross-linking employed during the purification process to obtain the 6JXR structure (Figure S1C) [12]. In addition, the CD3  $\zeta'$  chains of 8ES7 and of 7FJE both exhibit a nearly 7 Å shift of their C-termini compared to the C-terminus of CD3  $\zeta'$  in our TCR<sup>M1(58–66)</sup>/CD3 structure (Figs. 1G, 1I and S1C). The 7FJE TCR  $\beta$  chain carries gain-of-function (GOF) mutations (L281A/L285A) but TCR  $\beta$  chains of 8ES7 and our TCR<sup>M1(58–66)</sup>/CD3 structure carry WT L281 and L285. These GOF mutations have been found to affect the binding of a second cholesterol molecule [13], however, neither our structure nor 8ES7 showed density that match a second cholesterol molecule near the cytoplasmic side of the transmembrane domains in tentative cholesterol binding site 2 (CLR2) (Figure S1B). Both our structure and 8ES7 have non-cholesterol-shaped density occupying CLR2. Therefore, the observed CD3  $\zeta'$  shift may not be caused by those GOF mutations and the tentative second cholesterol binding site (CLR2) may be occupied by other lipids than cholesterol. Moreover, in our purification process, different from most other groups, we did not add cholesteryl hemisuccinate [12, 14, 15], but our structure still showed clear density shaped to match a cholesterol molecule near the extracellular side in cholesterol binding site 1 (CLR1), which indicates that this cholesterol molecule is derived from the native lipids in the cell membrane (Figure S1B). In conclusion, comparison of our TCR<sup>M1(58–66)</sup>/CD3 structure with TCR/CD3 structures in the literature confirms similar overall TCR/CD3 organization, while TCR/CD3 ectodomain N-linked glycosylation positions exhibit large variation. Moreover, our TCR/CD3 comparison indicates that native TCR/CD3 transmembrane domain organization is intrinsically dynamic and able to probe various conformations in combination with flexibility in lipid species coordination.

## Cryo-EM structure of pHLA-A\*02:01/TCR/CD3 complex

To gain insights into the molecular basis for full-length TCR<sup>M1(58–66)</sup>/CD3 recognition of the M1(58–66) peptide presented by HLA-A\*02:01 pMHCI (pHLA-A\*02:01<sup>M1(58–66)</sup>), we solved a cryo-EM structure of the pHLA-A\*02:01/TCR<sup>M1(58–66)</sup>/CD3 complex with overall resolution of 3.3 Å (Figs. 2A-2B and S7). The M1(58–66) peptide is binding to the HLA-A\*02:01  $\alpha$ 1 and  $\alpha$ 2 helices via multiple h-bonds and to the HLA-A\*02:01  $\beta$  sheet that forms the floor of the pHLA-A\*02:01 antigen-binding cleft through interactions such as h-bonds between the I2 backbone to the side chain of E63 and hydrophobic interactions between the I2 side chain to the side chains of HLA Y7, M45, V67, H70 and Y99. Also, we observed h-bonds formed between the backbone of T8 and the side chain of K146. The backbone of L9 forms h-bonds to the side chains of D77 and T143, and we observed hydrophobic contacts between the side chain of L9 and the side chains of Y116, L81 and W147 (Fig. 2C). The CDR loops of TCR  $\alpha$  and  $\beta$  chains make contacts with pHLA-A\*02:01<sup>M1(58–66)</sup> at a tilt angle ( $\Phi$ ) of 13.5° and rotation angle of 49.4° ( $\Theta$ ), resulting in a 5.5nm shift of the T cell membrane relative to the antigen-presenting cell (APC) and resulting in a separation of 12nm between the T cell and APC outer membranes at the position of receptor engagement. (Fig. 3A-3B) [25]. This result is in agreement with the reported physiological distance of T cells recognizing antigen presented by APC (Fig. 3B) [26]. TCR  $\alpha$  CDR loops interact with the M1(68 – 66) peptide with the S109 backbone through Van der Waal's contacts with G4 C $\alpha$ , whereas S37, R84 and Q113 of TCR  $\alpha$  interact with Q155, E154 and R65 of pHLA-A\*02:01<sup>M1(58–66)</sup> heavy chain, respectively. TCR  $\beta$  CDR loops, on the

other hand, form more contacts with peptide and HLA-A\*02:01. D37, Q57 and S110 of TCR  $\beta$  interact with the side chain of T8 and the backbone of G4 and V6 through h-bonds, respectively. Additionally, D37 interacts with HLA-A\*02:01 K146 and R109 and S113 interact with HLA-A\*02:01 Q155 via h-bonds; R109 inserts into a “notch” formed by HLA-A\*02:01 A150, V152, Q155, TCR  $\beta$  R109, S110, S113, Y114 and M1(58–66) F5 and F7 (Fig. 4C). TCR  $\alpha$  CDR loops form fewer interactions with pHLA-A\*02:01<sup>M1(58–66)</sup> than TCR  $\beta$  CDR loops with 218Å<sup>2</sup> and 418Å<sup>2</sup> buried surface area, respectively; additionally, they share a 327Å<sup>2</sup> buried surface area (Fig. 4D). The contacts of TCR  $\alpha$  and  $\beta$  with the M1(58–66) peptide are mostly identical to those observed in the previously solved crystal structures [17, 27]. We compared our bound pHLA-A\*02:01<sup>M1(58–66)</sup>/TCR<sup>M1(58–66)</sup>/CD3 with four previously published structures of pMHC-ligated full-length TCRs (Figure S2), aligning on the whole TCR. We compared calculated TCR-to-pMHC center of mass angular parameters ( $\theta$  and  $\varphi$ ) across these structures (Figure S2A-S2B), showing significant differences in pMHC engagement vector between these complexes.

Additionally, we compared the relative conformation of transmembrane helices in our structure with each of these published pMHC/TCR/CD3 complexes (Figure S2C-S2F). Our helices are closest in conformation with those reported by Susac *et al.* (Figure S2C; PDB 7PHR), with notable differences to the conformation of the CD3  $\zeta'$  chain compared to both structures from Saotome *et al.* (Figure S3D-S3E; PDBs 8ES8, 8ES9), with a CD3  $\zeta'$  C $\alpha$ -RMSD of 3.5 Å for PDB 8ES9 and 3.0 Å for PDB 8ES8. The helices in the pMHC-bound structure from Notti *et al.* (Figure S3F; PDB 9C3E) are all slightly shifted, with a mean helix C $\alpha$ -RMSD of 3.9 Å compared to our structure. Overall, our structure displays differences in pMHC engagement vector and some variability in transmembrane helix orientation when compared with existing full-length TCR structures.

Next, we compared our apo-TCR<sup>M1(58–66)</sup>/CD3 and bound pHLA-A\*02:01<sup>M1(58–66)</sup>/TCR<sup>M1(58–66)</sup>/CD3 structures (Figure S3). Although the overall C $\alpha$  RMSD is only 0.67 Å, there is still notable movement among different regions across various chains. Since CDR loops of TCR  $\alpha$  and  $\beta$  chains engage in interactions with pHLA-A\*02:01<sup>M1(58–66)</sup>, we expect the CDR loops to undergo movement and thus, in these regions, we observe RMSDs ranging from 2.4 Å to 3.4 Å (Figure S3A). The CD3 subunit ectodomains remain rigid and in place except for peripheral loops of the two CD3  $\epsilon$  chains. Both CD3  $\epsilon$  and  $\epsilon'$  G68-D71 loops undergo movement resulting in loop reshaping (Figure S3A-S3C). Our unsharpened density maps confirm placement of these loop C $\alpha$  traces of both apo-TCR<sup>M1(58–66)</sup>/CD3 and bound pHLA-A\*02:01<sup>M1(58–66)</sup>/TCR<sup>M1(58–66)</sup>/CD3 (Figure S4). CD3  $\zeta$  chains are critical for T cell signal transduction, but we observe only minimal movement of the ectodomains and nearly rigid transmembrane regions of CD  $\zeta$  (Figure S3A, S3E). We also noticed minor movement of the cytoplasmic transmembrane  $\alpha$ -helix terminus of CD3  $\delta$  (1.7Å for C $\alpha$ -D129), CD3  $\zeta$  (1.7Å for C $\alpha$ -R57), CD3  $\epsilon$  (2.7Å for C $\alpha$ -K156) and CD3  $\zeta'$  (2.3Å for C $\alpha$ -F55), and the largest movement was observed for TCR  $\beta$  (3.5Å for C $\alpha$ -K302) (Figure S3A, S3E).

## Lipidomics and LipIDens Analysis

During model building into our two cryo-EM maps (apo-TCR<sup>M1(58-66)</sup>/CD3 and pHLA-A\*02:01<sup>M1(58-66)</sup>/TCR<sup>M1(58-66)</sup>/CD3), we noticed distinct but ambiguous lipid density at three sites in the membrane-bound portion of the TCR structure. One of them aligns with a previously published cholesterol binding site, CLR1 [12, 13, 15] (Figure S1B); however, the other two lipid densities were not previously reported (lipid density 1 and lipid density 2) (Fig. 5). Lipid density 1 is located in the cavity between TCR $\alpha$ , TCR $\beta$ , CD3 $\delta$  chains near the extracellular side in both apo-TCR<sup>M1(58-66)</sup>/CD3 and pHLA-A\*02:01<sup>M1(58-66)</sup>/TCR<sup>M1(58-66)</sup>/CD3 (Fig. 5A), whereas lipid density 2 is at the intracellular side between TCR $\alpha$ , CD3 $\delta$ , CD3 $\epsilon'$ , CD3 $\zeta$  and CD3 $\zeta'$  chains (Fig. 5B). To identify the unknown two lipids occupying these sites, we employed a two-pronged experimental and computational approach.

First, we performed mass spectrometry-based global lipidomic analysis of the membrane-bound TCR<sup>M1(58-66)</sup>/CD3 fraction. We assayed two fractions (one that was eluted before the apo-TCR<sup>M1(58-66)</sup>/CD3 complex during the Size Exclusion Chromatography purification step, and one that was eluted after the complex) as controls and one fraction where the apo-TCR<sup>M1(58-66)</sup>/CD3 complex was eluted (Fig. 6A-6B). Since our purification adds no exogenous lipids, all detected lipids must originate from the cell membrane. Lipid profiling detected a total of 179 lipid species among both control and sample fractions, in which 98% (175 out of 179) species were specific to the isolated protein sample fraction of the apo-TCR/CD3 complex and served as candidates for corresponding to novel lipid densities 1 or 2. Of these lipids, the most represented class was phosphatidylethanolamine (PE) with 46 species followed by phosphatidylinositol (PI) and ceramide (Cer) with 21 species each. Other represented lipid classes included lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidic acid (PA), hexosylceramide (Hex1Cer and Hex2Cer), sphingomyelin (SM), triglyceride (TG), and diglyceride (DG) (Fig. 6A-6B).

Next, we used an established computational workflow, LipIDens, to investigate lipid identity in the sites occupied by lipid densities 1 or 2 [24]. Briefly, LipIDens uses simulation data that is independent of experimental electron density to identify sites of lipid binding on the protein, which can then be compared with experimental density to assess fit of particular lipid classes by both residence time (calculated from simulation data) and fit to density (calculated by Q-score). Using our lipidomics data, we generated a model system for investigation using the LipIDens workflow. We began by constructing an atomic model of our TCR structure embedded in a lipid bilayer, replicating the experimental class identity of lipids by percentage (70% phosphatidylcholine [PC], 16.5% phosphatidylethanolamine [PE], 6% sphingomyelin [SM], 3% phosphatidylinositol [PI], 3% phosphatidylserine [PS], 0.5% ceramide [CE], 0.4% phosphatidylglycerol [PG]) (Fig. 6B).

We then carried out PyLipID analysis [28] on the resultant sets of trajectories, outputting two sets of candidate lipid binding sites for cross referencing with observed electron density. The first lipid site, lipid site1, was identified as a distinct lipid binding site in both replicates of our LipIDens simulations, with 72% (26 shared residues of 36 total residues) overlap in involved residues between the independent replicates. All overlapping residues were located on TCR $\alpha$ , TCR $\beta$  and CD3 $\delta$  chains. Near the extracellular

lipid bilayer surface, those residues are hydrophilic (e.g. Q241 on TCR $\alpha$ , E277 on TCR $\beta$  and E98, D100 on CD3 $\delta$ ) and hydrophobic residues are enriched towards the center of the lipid bilayer. This pattern aligns with the cryo-EM lipid density orientation where the hydrophilic head is positioned toward the extracellular surface of the transmembrane region whereas the hydrophobic tail is oriented towards the center of the transmembrane region (Fig. 6C). Given the agreement between replicates, and the clear presence of density in our maps, we proceeded with screening candidate lipids that could bind to this site. In both replicates, the phosphatidylinositol class (PI) had the longest residence time in the site when compared with other lipid classes (Fig. 6D). This is particularly notable, as PI comprised only 3% of available lipids (Fig. 6B), indicating that this is not an artifact of oversampling the most abundant species in simulation. We therefore conclude that PI is the most likely candidate lipid class to occupy this binding site. Also, two distinct candidates, PI (16:0/18:2) and PI (16:1/18:1), were detected in negative ion mode at 834.5258 m/z and confidently annotated (A and B grades) in the lipidomics analysis (Figure S5A). A representative PI lipid species side chain was fit into the density for this binding site, with a calculated Q-score of 0.54 for the portion of the chain contained inside density (Fig. 6C).

In contrast to these findings, lipid density 2 was not identified as a distinct site in either of the LipIDens replicates (Figure S5B). This lipid density shows movement between apo-TCR<sup>M1(58–66)</sup>/CD3 and pHLA-A\*02:01<sup>M1(58–66)</sup>/TCR<sup>M1(58–66)</sup>/CD3, which indicates a dynamic lipid binding site (Fig. 5B). In addition to these two identified lipid binding sites, we analyzed LipIDens results for the cholesterol binding site CLR1. It was only identified as a lipid binding site in one of the LipIDens replicates, while it was at the junction between several identified sites in the other replicate (Figure S5B). Since the global lipidomics analysis did not detect any cholesterol esters or free cholesterol, we did not model cholesterol in the simulations. However, it should be noted that our mass spectrometry platform was not capable of detecting low levels of free cholesterol because it is poorly ionized in electrospray ionization. This technical limitation may explain both the ambiguity in identification of the tentative CLR1 site from simulations not containing cholesterol. In conclusion, we identify PI as a likely occupant of the lipid density 1 site, whereas our analysis indicates that the lipid density 2 site exhibits dynamic movement and thus can most likely be occupied by various lipid species.

## MD simulation and SPR to predict binding to mutant influenza peptides

Having solved a structure of TCR<sup>M1(58–66)</sup>/CD3 bound to pHLA-A\*02:01<sup>M1(58–66)</sup>, we sought to determine the importance of contacts between the antigen peptide and TCR for affinity and recognition. To assess the stability of the observed contacts in a dynamic structural ensemble, we performed all-atom molecular dynamics simulations of the pHLA-A\*02:01<sup>M1(58–66)</sup>/TCR<sup>M1(58–66)</sup>/CD3 complex for 1 $\mu$ s in triplicate. We then calculated side chain chi-angle dihedral order parameters for each residue across the simulation, with higher order parameters indicating greater contact stability, and lower order parameters indicating decreased stability [29]. Since the TCR seems to primarily form contacts with the backbone rather than the side chains of the M1(58–66) peptide, we focused on chi order parameters for the

corresponding TCR  $\beta$  chain residues mentioned above. While D37, Q57 and R109 featured chi order parameters larger than 0.7, S110 had an order parameter of  $\sim 0.5$ , suggesting a less stable contact over the course of simulation (Figs. 7A and S6A). Previous studies have shown that the affinity kinetics between TCR<sup>M1(58–66)</sup> and pHLA-A\*02:01<sup>M1(58–66)</sup> were dramatically affected by D37, Q57 and R109 mutations, but not by S110 mutations [17]. Our findings align with previously published experimental data and indicate that the contact between S110 and V6 is less stable, and therefore less important for affinity and recognition, than the contacts between other pairs of TCR  $\beta$  chain and M1(58–66) backbone residues. Notably, although R109 on the TCR  $\beta$  chain is not directly involved in contacts with M1(58–66) peptide, it inserts into the aforementioned “notch” and is critical for interactions with the whole pHLA-A\*02:01<sup>M1(58–66)</sup> (Fig. 4C).

Additionally, we wanted to assess the cross-reactivity of our engineered TCR toward known naturally occurring mutant versions of the M1(58–66) peptide. Previous studies have reported that several M1 peptide mutations, including L3W, G4E, and F5L, significantly decrease the affinity of the JM22 TCR for the M1/HLA-A\*02:01 complex [17]. We examined available influenza sequences to identify mutations which were 1. identified in humans, 2. somewhat abundant, and 3. whose affinity toward the JM22 TCR was not previously characterized. Our final list of mutations included G1R, L3V, F5V, V6M, and T8S (Figure S6B). We decided to investigate these mutations using a tandem computational and experimental approach. Running 3x1 $\mu$ s molecular dynamics simulations of each mutant peptide bound to MHC in the absence of TCR, we used a previously published pose clustering method characterizing the influence of mutations on peptide dynamics in the MHC groove to predict the influence of each mutation on affinity [30]. Simultaneously, we expressed each of these pMHC complexes and conducted surface plasmon resonance (SPR) measurements of their binding to our soluble TCR<sup>M1(58–66)</sup> (Figs. 7B and S6C). In our simulations, we observed that the mutations G1R, L3V, and T8S displayed very similar profiles to the wild-type M1 peptide, with  $\sim 80\%$  of peptide poses occupying a similar structural cluster in each set of simulations (Fig. 7A-7B). We further note that the  $\beta$ -carboxyl group of TCR  $\beta$  D37 forms an h-bond with the side chain of T8; the T8S mutation does not change the local chemistry and most likely the hydrogen bond is preserved. In contrast, F5V and V6M both displayed significant deviation from the wild-type cluster, with only  $\sim 60\%$  of frames for F5V and  $\sim 20\%$  of frames for V6M occupying the wild-type cluster (Fig. 4B). We then compared these results with our SPR measurements, which showed similar affinity (23-78 $\mu$ M) for WT, G1R, L3V, and T8S peptides, but significantly reduced affinity ( $> 100\mu$ M) for both F5V and V6M (Figs. 7B and S6C). These results suggest that the F5V and V6M mutations perturb peptide dynamics in the MHC binding groove away from the consensus pose adopted by the wild-type peptide, and that this change in dynamics is reflected in significantly reduced affinity for the TCR<sup>M1(58–66)</sup>.

## Discussion

Our structural determination of both the apo- and pHLA-bound TCR<sup>M1(58–66)</sup>/CD3 complexes specific to the influenza A derived matrix M1(58–66) epitope provides molecular insights into viral-peptide-specific

TCRs. The TCR<sup>M1(58–66)</sup>/CD3 complex in this study was engineered by swapping JM22 TCR ectodomains into a healthy donor isolated TCR [12]. Our pHLA-bound TCR<sup>M1(58–66)</sup>/CD3 showed an overall canonical antigen docking angle, allowing us to measure T cell distance to APC presenting M1(58–66) peptide.

Previous studies showed that structural comparison of unliganded, healthy donor TCR/CD3 with liganded, cancer-related pMHC/TCR/CD3 did not reveal significant allostery upon ligation [14]. However, these comparisons are not generated using identical TCR/CD3 complexes and therefore may not demonstrate the native dynamics of TCR/CD3 complexes upon ligation. We aligned our apo-TCR<sup>M1(58–66)</sup>/CD3 and pHLA-A\*02:01<sup>M1(58–66)</sup>/TCR<sup>M1(58–66)</sup>/CD3 structures for our analysis. Notably, although the overall structure assembly remains unchanged, our liganded structure showed minor reorganization of the CD3ε chains – specifically at the G68-D71 loop region. In addition, we observed movement upon pMHC engagement of the transmembrane helices of CD3 δ, CD3 ε, CD3 ζ, CD3 ζ' and TCR β. These movements could result from the intrinsic flexibility of TCR/CD3 or may have functional relevance; further studies are needed to evaluate these observations.

Our structures, expressed and purified without exogenous cholesterol or other lipids, captured native lipid-binding events in a lipid binding pocket identified between TCRα/β and CD3δ chains (lipid density 1). LipIDens simulations, in combination with lipidomics, identified the phosphatidylinositol (PI) lipid class, particularly PI(16:0/18:2), as a high-probability candidate for occupancy of this lipid binding site. Although PI binding to membrane-bound TCR/CD3 has been previously reported [31], our site differs from the ones described in prior literature, suggesting that PI may interact at several sites in the transmembrane domains of TCR/CD3. Interestingly, we observed no defined density for the second tentative cholesterol binding site (CLR2) described in PDB 7FJD, nor clear preference for a particular lipid species suggesting that CLR2 may be a various lipid binding site. By cross-comparison with PDB 7FJD, 7FJE and 8ES7, the lack of stable CLR2 density and cytoplasmic side CD3ζ' movement imply that cholesterol is not a universal structural requirement but may participate in specific state transitions. These findings differ from a previous hypothesis from *Chen et al.* (2022), which concluded that lipid binding (notably cholesterol) in the CLR2 site contributes to TCR resting-state stabilization.

Our identification of a previously undescribed lipid binding pocket near the CD3δ chain warrants further investigation. It raises the possibility that site-specific lipidation may regulate conformational transitions within lipid nanodomains. Targeted mutagenesis of this pocket, or in situ lipid exchange experiments, could clarify its functional role. Moreover, while our work captured static snapshots, the dynamic nature of the CD3 transmembrane helices and cytoplasmic tails, especially in the apo versus ligand-bound states, suggests a role for force-induced or lipid-mediated modulation of signaling motifs. Future efforts could combine time-resolved cryo-electron tomography (cryo-ET) with live-cell mechanobiology and high-resolution mass spectrometry to define the causal chain from antigen recognition to ITAM exposure.

Our study also demonstrates the ability to predict experimental determinants of affinity using computational methods, in particular atomistic molecular dynamics simulations combined with surface

plasmon resonance. By identifying stable contacts formed by the TCR, we can avoid designing unwanted mutations that likely substantially reduce affinity, thereby narrowing the set of residues to consider for design. Conversely, positions without stable contacts provide an opportunity for design to substantially improve affinity, directing design toward a small set of candidate sites. This methodology therefore provides a useful set of positive and negative criteria for selecting mutation sites when engineering a TCR for affinity and target recognition.

Predicting the impact of peptide mutations on TCR affinity is also meaningful, especially in the context of infectious disease. While cancer neoantigen targeted therapy desires sequence specificity (i.e. binding to mutant peptide but not wild-type peptide), virus targeted therapy would likely benefit from sequence recognition breadth over specificity (i.e. the ability to bind many mutant peptides from the same viral protein). Our simulation methodology allows us to generate relative affinity predictions for mutant peptides based on differences in peptide dynamics on the MHC groove. We were able to confirm our predictions with SPR measurements, suggesting that this methodology may be able to generalize to other systems.

In summary, our tandem computational and experimental methods offer a platform for predicting the importance of particular TCR-pMHC contacts for affinity, and for predicting the cross-reactivity of mutant peptides toward a given TCR. Together, our findings serve as a proof-of-concept for structural determination of engineered TCR/CD3 complexes and demonstrate that the combination of experimental and computational methodologies can identify lipid binding and predict determinants of affinity in the context of full-length TCR/CD3.

## Materials and methods

### Construct design

Genes encoding full-length sequences of CD3 subunits were synthesized and sub-cloned in pTwist-CMV-Puro vector (Twist Bioscience). Each of the CD3 subunits were separated by 2A-peptide consensus motifs (P2A, E2A and T2A). A GFP expression gene and a twin-strep tag gene were cloned after CD3 $\zeta$  linked by a 3C cleavage site. Genes encoding M1(58–66) specific TCR $\alpha$  and TCR $\beta$  variable domains were synthesized and replaced into a published full-length TCR sequence [12, 17]. The full-length sequences encoding TCR $\alpha^{M1(58-66)}$  and TCR $\beta^{M1(58-66)}$  chains were sub-cloned into the mammalian expression vector pαH and separated by a 2A-peptide consensus motif (E2A). HLA-A\*02:01 and  $\beta$ 2m constructs were sub-cloned into pET-29b vectors for E. coli expression. Single-chain trimer (SCT) pHLA-A\*02:01 was designed as peptide, HLA-A\*02:01 and  $\beta$ 2m into a single open reading frame followed by 6x His tag and Avi tag as previously described [32], then subcloned into pVax1 plasmid. Soluble TCR $M1(58-66)$  (sTCR) was designed by fusing variable TCR $\alpha$  and TCR $\beta$  into human IgG Fc with knob-hole mutations [33]. sTCR gene was subcloned into pVax1 plasmid.

# Protein expression and purification

Expi293 cells (Invitrogen) were cultured in Expi293 culture medium (Invitrogen). TCR<sup>M1(58–66)</sup>/CD3 was expressed by transient transfection using Fecto Pro (Polyplus) according to the manual. 1L of Expi293 cells were transfected and incubated at 37°C and 5% CO<sub>2</sub> for 24 hours. 11mM valproic acid sodium was added into the culture and incubated at 37°C and 5% CO<sub>2</sub> for another 24 hours. Transfected cells were harvested by centrifugation and washed by cold PBS three times. Cell pellets were stored at -80°C for future use.

For the pHLA-A\*02:01<sup>M1(58–66)</sup> complex used in our structural studies, HLA-A\*02:01 heavy chain and β2m cloned in pET29b plasmids were transformed into BL21 E. coli chemically competent cells. Transformed cells were induced with 0.2mM IPTG at 37°C when density reached OD 0.6 and were harvested after 12 hours. The harvested cell pellets were resuspended in TBS, sonicated, and centrifuged at 12,000g to remove soluble impurities in the supernatant. The resulting pellets comprising inclusion bodies were stored at -80°C for future use. For refolding, inclusion body pellets of heavy chain and β2m were thawed, dissolved in 8M urea and centrifuged at 12,000g to remove membrane impurities. Protein was then reduced by addition of 1mM DTT and incubated for an hour to break all the disulfide bonds before refolding. 10μM peptide, 2μM β2m and 1μM heavy chain were sequentially diluted into 200mL of 0.4M Arg-HCl, 0.1M Tris-HCl pH8, 5mM reduced glutathione, 0.5mM oxidized glutathione, 2mM EDTA, 0.5mM PMSF. After three days of refolding, the product was harvested, concentrated in 30kD Amicon Ultra-15 centrifugal tubes, and purified by size exclusion chromatography (SEC) using a 10/300 Superdex 200 Increase column (Cytiva). Fractions of pHLA-A\*02:01<sup>M1(58–66)</sup> were combined, concentrated by 30kD Amicon Ultra-15 centrifugal tubes, snap-frozen, and stored at -80°C for future use.

For purification of apo-TCR<sup>M1(58–66)</sup>/CD3 protein, cell pellets were thawed and directly resuspended in solubilization buffer (50mM Tris-HCl, pH 8, 150mM NaCl, 1% GDN) (Anatrace). Cells were solubilized for 2 hours followed by ultracentrifugation at 150,000g for 1.5 hours. Supernatant was clarified and incubated with Streptactin Superflow plus resin (Qiagen) for 1.5 hours at 4°C. After the resin was washed by wash buffer (50mM Tris-HCl, pH 8, 150mM NaCl, 0.02% GDN), protein was eluted by addition of 10mM biotin in the wash buffer. Subsequent size exclusion chromatography was performed on a Superose 6 increase column (Cytiva) in wash buffer. Fractions containing TCR<sup>M1(58–66)</sup>/CD3 were concentrated to 1.8mg/mL and stored for complexation with pHLA-A\*02:01<sup>M1(58–66)</sup>.

For pHLA-A\*02:01 protein carrying different M1(58–66) mutant peptides, SCT plasmid was co-transfected with a BirA expression plasmid into Expi293 cells for in vivo biotinylation. Supernatant was collected after 5 days and purified through an IMAC affinity column (Cytiva) followed by size exclusion chromatography on a 10/300 Superdex 200 increase column (Cytiva) in PBS.

For soluble TCR<sup>M1(58–66)</sup> protein production, our sTCR expression plasmid was transiently transfected into Expi293 cells. Supernatant was collected after 6 days and purified through a Protein A affinity column (Cytiva).

# Cryo-electron microscopy sample preparation and data collection

UltrAuFoil R1.2/1.3 grids were glow discharged and used for cryo-EM sample preparation. A Vitrobot Mark IV (Thermo Fisher Scientific) was pre-cooled to 4°C at 100% humidity. For the pHLA-A\*02:01<sup>M1(58-66)</sup>/TCR<sup>M1(58-66)</sup>/CD3 sample, 10ul of pHLA-A\*02:01<sup>M1(58-66)</sup> at 1.8mg/mL and 10ul of apo-TCR<sup>M1(58-66)</sup>/CD3 at 1.8mg/mL were mixed on ice for 30 minutes prior to freezing. 4uL of apo-TCR<sup>M1(58-66)</sup>/CD3 or pHLA-A\*02:01<sup>M1(58-66)</sup>/TCR<sup>M1(58-66)</sup>/CD3 sample was applied to the grid followed by plunge freezing in liquidized ethane cooled by liquid nitrogen.

Grids were clipped and loaded into a Titan Krios electron microscope equipped with a Gatan K3 camera (Thermo Fisher Scientific). Cryo-EM movies were collected at a nominal magnification of 105,000x with defocus range of -0.5 to -2.5μm. The energy filter was tuned for a 20eV slit width. 5032 movies were collected for the apo-TCR<sup>M1(58-66)</sup>/CD3 sample and 6322 movies were collected for pHLA-A\*02:01/TCR<sup>M1(58-66)</sup>/CD3.

## Cryo-EM data processing

Apo-TCR<sup>M1(58-66)</sup>/CD3 and pHLA-A\*02:01<sup>M1(58-66)</sup>/TCR<sup>M1(58-66)</sup>/CD3 data sets were processed in Relion and CryoSPARC similarly. Movie frame alignment and weighted signal summation were performed and followed by CTF estimation [34]. Relion autopicker was used on a small portion of the data sets followed by 2D image classification to generate templates. Relion template-based picking was then used on the full data sets followed by several rounds of 2D image classification [35]. Ab initio 3D reconstruction was performed in CryoSPARC using the particles selected from 2D image classification followed by heterogeneous refinement [36]. Particles aligned in one of the best heterogeneous refinements were selected for non-uniform refinement in CryoSPARC. CTF refinement, beam-tilt correction and Bayesian particle polishing were performed in Relion following by another round of non-uniform refinement in CryoSPARC.

## Model building and refinement

Both apo-TCR<sup>M1(58-66)</sup>/CD3 and pHLA-A\*02:01<sup>M1(58-66)</sup>/TCR<sup>M1(58-66)</sup>/CD3 models were built in Coot guided by our density maps [37]. Initial models were generated based on PDB 6XJR for apo-TCR<sup>M1(58-66)</sup>/CD3 and PDB 6XJR and 2VLJ for pHLA-A\*02:01<sup>M1(58-66)</sup>/TCR<sup>M1(58-66)</sup>/CD3. TCR α and β variable domains were replaced from PDB 2VLJ. Refinements were performed with Rosetta FastRelax. Geometry and fit-to-map were evaluated with MolProbity, Privateer and EMRinger [38]. UCSF chimera and UCSF chimeraX were used to visualize the models and maps [39].

## Lipidomics

Samples were obtained from the size exclusion chromatography purification. Lipids were extracted using the Folch method (2:1 v/v chloroform/methanol), repeated twice. Extracts were dried under nitrogen,

resuspended in 35  $\mu$ L mobile phase B (see below), and stored at  $-20^{\circ}\text{C}$  prior to LC-MS/MS analysis. Samples (5  $\mu$ L per injection) were analyzed by LC-MS/MS on a Thermo Scientific Q Exactive HF-X mass spectrometer coupled to a Vanquish Horizon UHPLC System. Data were acquired separately for positive and negative polarities. LC separation was performed by reversed-phase chromatography using a Thermo Scientific Accucore C30 column (2.1 mm x 150 mm, 2.6  $\mu$ m particle size) with 50:50 v/v acetonitrile/water (mobile phase A) and 88:10:2 v/v/v isopropanol/acetonitrile/water (mobile phase B), both containing 5 mM ammonium formate and 0.1% formic acid. MS1 scans were acquired at 120k resolution, and data-dependent MS2 scans were acquired for the top 20 ions at 15k resolution with 0.4 m/z isolation width and stepped NCE 20/30/40. Lipid species were identified based on MS/MS fragmentation compared to an in-silico database of > 1.7 million species based on known fragmentation of lipids and quantified by peak area using ThermoScientific LipidSearch 4.2. Raw data were filtered by confidence of identification (grade), expected adducts for each lipid class, and peak quality parameters. Certain lipid species were filtered from the dataset to improve data quality (e.g., retention time outliers). Relative quantitation was based on equivalent amount of sample injected.

## LipIDens simulations

LipIDens simulations were carried out as previously described using the LipIDens[24] and PyLipID[28] workflows. Briefly, a coarse-grained model of the TCR embedded in a bilayer membrane was constructed using MARTINI version 2.2 coarse-grained force-field parameters. Lipidomics data was used to guide the percentage of each class of lipids included in both membrane leaflets (70% phosphatidylcholine [PC], 16.5% phosphatidylethanolamine [PE], 6% sphingomyelin [SM], 3% phosphatidinositol [PI], 3% phosphatidylserine [PS], 0.5% ceramide [CE], 0.4% phosphatidylglycerol [PG]). Systems were configured in a cubic 200 $\text{\AA}$  box, with 2 independent runs of 5 replicates. Energy minimization was carried out for 1000 steps using the Verlet cutoff scheme, followed by 1ns of equilibration using a 2fs timestep and 10ns of equilibration using a 10fs timestep. Production runs were carried out in the NPT ensemble for 15 $\mu$ s at 310K using a velocity rescaling thermostat and a Parrinello-Rahman barostat with a 10fs timestep. PyLipID was used to identify binding sites using a lower cutoff of 0.5 and an upper cutoff of 0.7. Sites were correlated with experimentally observed electron density, and the class with longest residence time across both independent runs was identified as the highest probability lipid for binding to that site. Q-score was calculated for a fit PI pose using ChimeraX.

## All-atom MD simulations

All-atom molecular dynamics simulations in explicit solvent were carried out using GROMACS version 2023.2, employing the AMBER99SB\*-ILDN protein force field and TIP3P water model [40]. LINCS and SETTLE constraint algorithms were used to constrain peptide/protein and water molecules, respectively. An integration time step of 2fs was used with coordinates output every 10ps. Short-range interactions were treated with a Verlet cutoff scheme with 10 $\text{\AA}$  electrostatic and Van der Waals cutoffs, and long-range electrostatics were treated with the particle mesh Ewald (PME) method with a grid spacing of 1.2 $\text{\AA}$  and cubic interpolation. Simulations were performed in cubic simulation boxes with 10 $\text{\AA}$  solvent padding, and periodic boundary conditions were used in all three spatial dimensions. The

thermodynamic ensemble was isothermal-isobaric (NPT) with the temperature being kept constant at 300K by a velocity rescaling thermostat with a stochastic term with 0.1ps time constant, and pressure kept constant at 1bar pressure using an isotropic Berendsen barostat with 0.5ps time constant and  $4.5 \times 10^{-5} \text{ bar}^{-1}$  isothermal compressibility.

The pHLA-A\*02:01<sup>M1(58–66)</sup>/TCR<sup>M1(58–66)</sup>/CD3 structure served as the primary input with the HLA-A\*02:01  $\alpha 3$  domain and  $\beta 2m$ , as well as the TCR constant and transmembrane domains, removed to speed up simulation. Each system was solvated to overall neutral charge and contained  $\text{Na}^+$  and  $\text{Cl}^-$  ions to yield a concentration of 0.15M. After 500 steps of steepest-descent energy minimization, initial velocities were generated at 65K with linear heating up to 300K over 2ns. Three independent trajectories for each peptide-loaded HLA-A:02:01, TCR, and pHLA-A\*02:01/TCR complex were acquired for 1 $\mu$ s using the Wistar Institute HPC computational cluster. Analysis was performed using GROMACS and the Visual Molecular Dynamics (VMD) package. Visualizations were produced with PyMOL (v2.5.5) [41]. For analysis, periodic boundary conditions were removed, and 1000 equally spaced frames from each trajectory (3000 frames in total for three independent replicates) were extracted using gmx trjconv. Phi/psi/chi angles and dihedral order parameters were calculated using gmx chi.

## Surface Plasmon Resonance

Surface plasmon resonance (SPR) experiments were performed using a low-charge-density polycarboxylate hydrogel sensor chip (HLC1000M, Xantec Bioanalytics) to minimize nonspecific binding arising from the high protein concentrations used in the assay. Neutravidin was immobilized on the chip surface by standard amine-coupling chemistry according to the manufacturer's instructions. Subsequently, 85–110 response units (RU) of each biotinylated pMHCI complex were captured on the Neutravidin-coated surface. Binding experiments were conducted in running buffer containing 10 mM HEPES (pH7.4), 150 mM NaCl, and 0.05% Tween-20. Soluble TCR (sTCR) was prepared as a 1:2 serial dilution series beginning at 16 $\mu$ M, generating a total of seven concentrations in running buffer. Samples were injected over the sensor surface with an association phase of 90s followed by a dissociation phase of 300s.

## Declarations

## Competing interests

All authors declare no completing interests.

## Author contributions

J.D., S.E.G and J.P. conceptualized and designed the project. J.D., S.E.G., K.B., W.Z., J.C., S.G., A.R.G. and J.P. performed, designed and analyzed experiments. J.D. and J.P. performed, processed and analyzed

cryo-EM experiments. J.D., K.B. and J.C. expressed and purified proteins. J.D., S.E.G. and S.G. performed molecular dynamics computational work. W.Z. and A.R.G. performed and analyzed lipidomics experiments. J.P. supervised and coordinated the study.

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## Figures

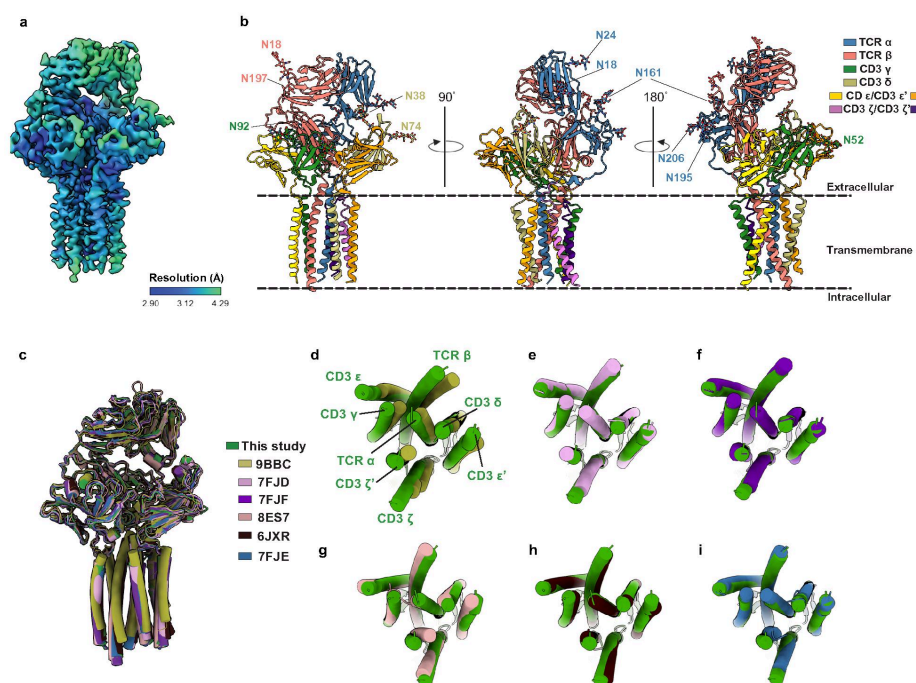


**Fig. 1 Cryo-EM structure of apo-TCR<sup>M1(58-66)</sup>/CD3.** **a.** Overview of cryo-EM density map of apo-TCR<sup>M1(58-66)</sup>/CD3. Density map is colored according to local resolution. **b.** Three views of apo-TCR<sup>M1(58-66)</sup>/CD3 atomic model. TCR  $\alpha$ , TCR  $\beta$ , CD3  $\delta$ , CD3  $\epsilon$ , CD3  $\epsilon'$ , CD3  $\gamma$ , CD3  $\zeta$ , CD3  $\zeta'$  are colored as blue, salmon, khaki, gold, orange, green, light purple and deep purple, respectively. N-linked glycans are shown as stick. TCR  $\alpha$  and  $\beta$  chains are numbered using IMGT numbering scheme. **c.** Structural comparison of apo-TCR<sup>M1(58-66)</sup>/CD3 (green) with multiple published TCR/CD3 complexes. Extracellular domains are displayed as ribbon cartoon and the transmembrane domains are displayed as rolls. All models were superimposed with apo-TCR<sup>M1(58-66)</sup>/CD3. **d-i.** Each PDB model compared to our apo-TCR<sup>M1(58-66)</sup>/CD3 structure (green). PDB 9BBC, 7FJD, 7FJF, 8ES7, 6JXR and 7FJE are colored as deep khaki, light purple, deep purple, dusty rose, black and blue, respectively.

## Figure 1

See image above for figure legend.

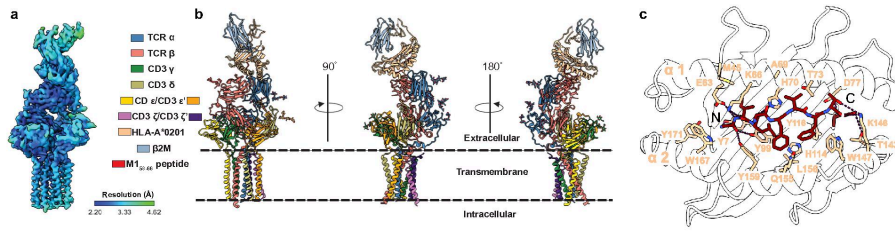


**Fig. 2 Cryo-EM structure of pHLA-A\*02:01<sup>M1(58-66)</sup>/TCR<sup>M1(58-66)</sup>/CD3.** **a.** Cryo-EM density map of our pHLA-A\*02:01<sup>M1(58-66)</sup>/TCR<sup>M1(58-66)</sup>/CD3 complex. Density map is colored according to local resolution. **b.** Three views of pHLA-A\*02:01<sup>M1(58-66)</sup>/TCR<sup>M1(58-66)</sup>/CD3 atomic model. TCR α, TCR β, CD3 δ, CD3 ε, CD3 ε', CD3 γ, CD3 ζ, CD3 ζ' are colored as blue, salmon, khaki, gold, orange, green, light purple and deep purple, respectively. HLA-A\*02:01, β2M and M1(58-66) peptide are colored as light tan, light blue and red. **c.** M1(58-66) peptide binding to HLA-A\*02:01. HLA-A\*02:01 is displayed as cartoon and the amino acids involved in the interaction are displayed as stick. M1(58-66) peptide backbone and side chain is displayed as stick. H-bonds are displayed as dashed line and colored as purple.

## Figure 2

See image above for figure legend.

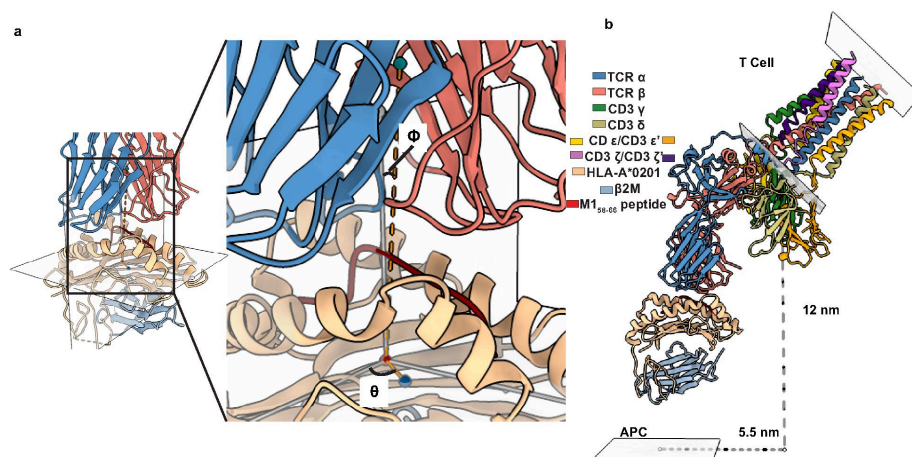


**Fig. 3 Analysis of TCR<sup>M1(58-66)</sup>/CD3 engaging pHLA-A\*02:01<sup>M1(58-66)</sup>.** **a.** Geometry of TCR<sup>M1(58-66)</sup>/CD3 binding to pHLA-A\*02:01<sup>M1(58-66)</sup>. The tilt angle ( $\Phi$ ) and rotation angle ( $\Theta$ ) were measured between the binding groove center of mass (coordinate axes origo) to the TCR V $\alpha$ /V $\beta$  center of mass (upper green sphere) and its projection to the horizontal plane. **b.** Estimated distances between T cell recognizing antigen presenting cell. Distances were measured in ChimeraX.

## Figure 3

See image above for figure legend.

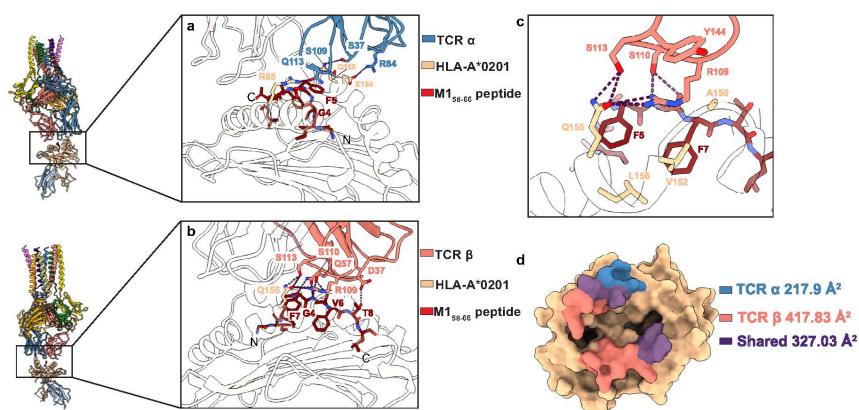


**Fig. 4** pHLA-A\*02:01<sup>M1(58-66)</sup>/TCR<sup>M1(58-66)</sup>/CD3 complex binding interface. **a.** Interactions of TCR α CDR loops to M1(58-66) peptide and h-bonds to HLA-A\*02:01 heavy chain. **b.** Interactions of TCR β CDR loops to M1(58-66) peptide and h-bonds to HLA-A\*02:01 heavy chain. H-bonds are colored as chocolate dashed lines. TCR α and β chains are numbered in IMGT numbering scheme. **c.** A “notch” formed by HLA-A\*02:01 A150, V152, Q155, TCR β R109, S110, S113, Y114 and M1(58-66) F5 and F7. **d.** Buried surface areas of HLA-A\*02:01 heavy chain contacting TCR α (blue) and β (salmon). The shared buried surface area between TCR α and β chains is colored as purple.

## Figure 4

See image above for figure legend.

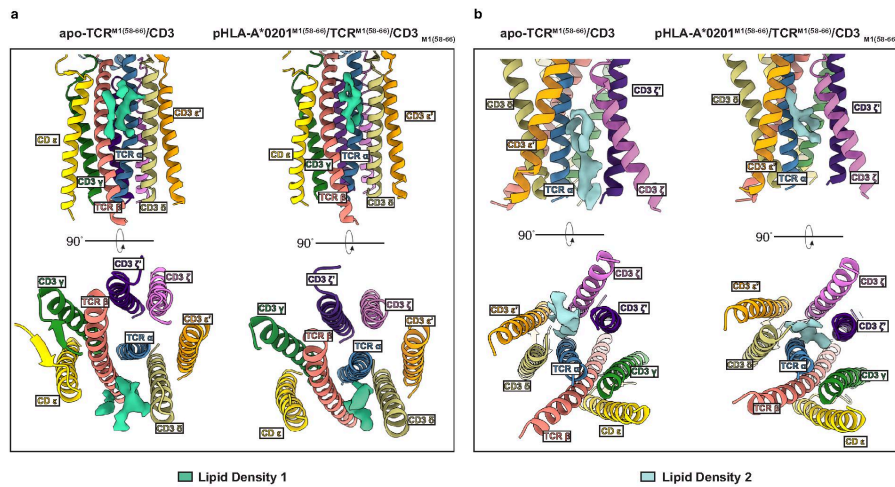


**Fig. 5** Two lipid densities identified in both apo-TCR<sup>M1(58-66)</sup>/CD3 and pHLA-A\*02:01<sup>M1(58-66)</sup>/TCR<sup>M1(58-66)</sup>/CD3. Lipid density 1 is colored as forest green and lipid density 2 is colored as light aqua.

**a.** Lipid density 1 position in apo-TCR<sup>M1(58-66)</sup>/CD3 model (left) and pHLA-A\*02:01<sup>M1(58-66)</sup>/TCR<sup>M1(58-66)</sup>/CD3 model (right). Top panels show front views of lipid density 1. Bottom panels show intracellular view of lipid density 1. **b.** Lipid density 2 position in apo-TCR<sup>M1(58-66)</sup>/CD3 model (left) and pHLA-A\*02:01<sup>M1(58-66)</sup>/TCR<sup>M1(58-66)</sup>/CD3 model (right). Top panels show front view of lipid density 2. Bottom panels show intracellular view of lipid density 2.

## Figure 5

See image above for figure legend.

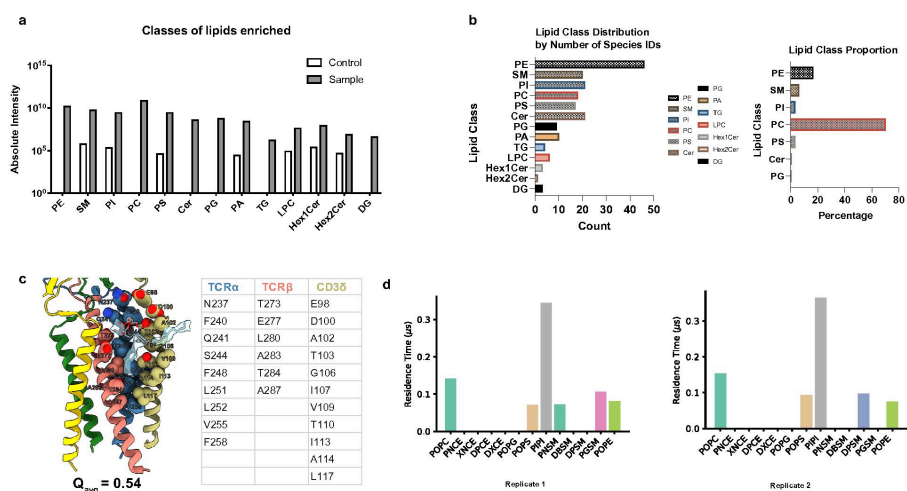


**Fig. 6 Lipidomics and LipIDens analysis of observed lipid densities. a-b.** Lipidomics result from LC-MS. PE: Phosphatidylethanolamine, SM: Sphingomyelin, PI: Phosphatidylinositol, PC: Phosphatidylcholine, PS: Phosphatidylserine, Cer: Ceramide, PG: Phosphatidylglycerol, PA: Phosphatidic acid, TG: Triglyceride, LPC: Lysophosphatidylcholine. Hex1Cer: Simple Glc series (Ceramide with 1 hexose), Hex1Cer Simple Glc series (Ceramide with 2 hexose), DG: Diglyceride. **a.** All lipids detected in control and sample plotted based on absolute intensity of each class. **b.** All detected lipid class distributions plotted by number of species IDs of each class and proportions of each class. **c-d.** LipIDens result for lipid density 1. **c.** Repeat residues of apo-TCR<sup>M1(58-66)</sup>/CD3 chains identified in our two LipIDens replicate simulations. Residues are displayed as balls. Repeat residues from each chain are listed on the right. TCR  $\alpha$  and  $\beta$  chains are numbered in IMGT numbering scheme. Representative PI species (C 18:0/20:4) fit into density for lipid site 1, with Q-score. Density is colored in blue with transparency. **d.** Two replicates of this density position. Binding site residence time ( $\mu$ s) of each lipid that bound at this position.

**Figure 6**

See image above for figure legend.

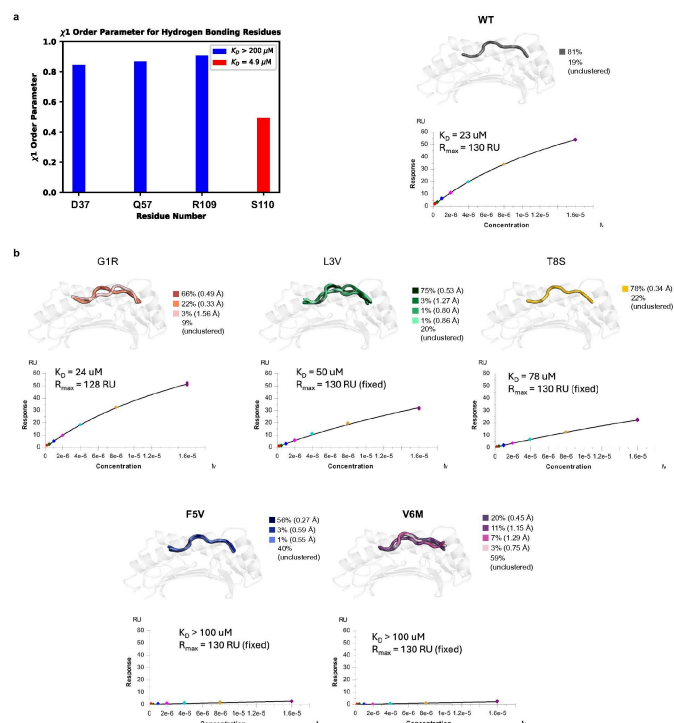


**Fig. 7 Analysis of TCR and M1(58-66) peptide mutation influence on TCR<sup>M1(58-66)</sup> affinity by MD simulation and SPR. a.** Calculated  $\chi_1$  order parameters for representative hydrogen-bonding residues, colored by impact of mutation to alanine on TCR affinity **b.** Peptide backbone clusters generated using a backbone root mean squared deviation (RMSD) cutoff of 0.3Å. The percentage of frames corresponding to each cluster relative to the total number of frames across a 3x1μs MD simulation is noted for all clusters representing at least 1% of frames. The backbone RMSD between the centroid of each cluster and the centroid of the predominant WT cluster is noted in parentheses. The HLA-A\*02:01 groove is shown in light gray for reference. Corresponding SPR measured for each mutated pHLA-A\*02:01 to TCR is presented under the stimulation representatives.

## Figure 7

See image above for figure legend.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1statistics.xlsx](#)
- [SupplementaryInformation.pdf](#)