The Structural Basis for Membrane Assembly of Immunoreceptor Signaling Complexes

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Abstract

Immunoreceptors are TM complexes that consist of separate ligand-binding and signal-transducing modules. Mounting evidence suggests that interactions with the local environment may influence the architecture of these TM domains, which assemble via crucial sets of conserved ionisable residues, and also control the peripheral association of immunoreceptor tyrosine-based activation motifs (ITAMs) whose phosphorylation triggers cytoplasmic signalling cascades. We now report a molecular dynamics (MD) simulation study of the archetypal T-Cell Receptor (TCR) and its cluster of differentiation 3 (CD3) signalling partners, along with the analogous DNAX-activation protein of 12 kDa (DAP12) / Natural Killer group 2C (NKG2C) complex. Based on >15 µs of explicitly solvated, atomic-resolution sampling, we explore molecular aspects of immunoreceptor complex stability in different functionally relevant states. A novel alchemical approach is used to simulate the cytoplasmic CD3ε tail at different depths within lipid bilayer models, revealing that the conformation and cytoplasmic exposure of ITAMs are highly sensitive to local enrichment by different lipid species and to phosphorylation. Furthermore, simulations of the TCR and DAP12 TM domains in various states of oligomerization suggest that, during the early stages of assembly, stable membrane insertion is facilitated by the interfacial lipid/solvent environment and/or partial ionization of charged residues. Collectively, our results indicate that the architecture and mechanisms of signal transduction in immunoreceptor complexes are tightly regulated by interactions with the microenvironment.
Introduction

Immunoreceptors are multi-component membrane proteins that assemble via their transmembrane (TM) domains in the endoplasmic reticulum (ER), prior to being exported to the cell surface. Typically, the ligand recognition and signal-transducing functions in such complexes are performed by separate chains; the latter are effected via signaling modules such as ITAM motifs. ITAMs contain tyrosines within the sequence YxxL/I, and typically appear within cytoplasmic tails as pairs separated by ~6-8 residues. Upon receptor activation, the tyrosines of ITAMs become exposed to phosphorylation by Src family tyrosine kinases to initiate downstream signaling cascades. The archetypal ITAM-based immunoreceptor complex is the TCR-CD3 complex, which plays an essential role in adaptive immunity against infectious agents, by activating the T-cell in response to antigen presentation by MHC molecules (Love & Hayes, 2010). The TCR is composed of two disulfide-bridge connected chains, α and β, each of which is composed of an extracellular segment containing immunoglobulin domains involved in antigen recognition, along with a TM domain, and short cytoplasmic tail (Wucherpfennig et al, 2009). The TCRαβ complex non-covalently associates with the TM domains of CD3 molecules, including CD3εγ, CD3εδ, and (disulfide-bridge connected) CD3ζζ dimers, each of whose cytoplasmic tails contains one or more ITAMs for signal transduction. Numerous other such immunocomplexes also exist, with analogous rules of assembly; for example, ITAM-containing DAP12 is a disulfide-bonded homodimer that forms the signaling component of numerous activating receptors such as NKG2C / CD94. It is still unclear how extracellular antigen binding is transduced into conformational changes that lead to release of cytoplasmic ITAM tails (Bezbradica, J.S. & Medzhitov, 2012); recent evidence suggests that activation may result in alterations between bent and extended states of the TCRα TM domain (Brazin et al, 2018), and changes in the
separation of the juxtamembrane regions of the CD3ζζ (Lee et al, 2015), thereby altering associations between subunits within the TCR.

A multitude of biochemical and mutational studies have revealed that electrostatic interactions between conserved ionisable residues within the TM domains of immunoreceptors are key to their assembly (Manolios et al, 1991; Bonifacino et al, 1991; Zidovetzki et al, 1998; Garrity et al, 2005). Call, Wucherpfennig and co-workers developed methods to isolate intact radiolabeled protein complexes along the assembly pathway, revealing that it proceeds via three 3-helix assembly steps: an acidic residue in the D/E-XX-T motif of each CD3 heterodimer enables them to progressively associate with one of three basic residues on a single TCRαβ heterodimer (Call et al, 2002; Call & Wucherpfennig, 2004; Call et al, 2005; Feng et al, 2005). This has emerged as the central organizing principle in TM immunoreceptor complex architecture. Similarly, electrostatic interactions between cytoplasmic tails and plasma membrane lipids appear to be crucial for the control of ITAM phosphorylation (Wu et al, 2015). Biophysical experiments have revealed that the free tails are intrinsically disordered but become structured upon binding to membranes containing anionic lipids (Duchardt et al, 2007; Aivazian & Stern, 2000; Xu et al, 2008), mediated in part by polybasic regions near to the ITAM motifs (Deford-Watts et al, 2009; Deford-Watts et al, 2011; Xu et al, 2008; Zhang et al, 2011; Yang et al, 2017), thereby rendering the tyrosines inaccessible to phosphorylation. This may be regulated by changes in local enrichment of anionic lipids (Gagnon et al, 2012) or calcium ion concentrations (Yang et al, 2017).

Several studies have helped to provide a structural context to immunoreceptor function. The solution NMR structure of the CD3ζζ TM domains in detergent micelles revealed a left-handed parallel α-helical dimer, with the crucial aspartate sidechains facing one another, and
a tyrosine/threonine pair forming additional stabilizing inter-helical hydrogen bonds (Call et al, 2006). The presence of a unique intermolecular nuclear overhauser effect (NOE) signal to water suggested that waters may play a structural role in stabilisation of the acidic group at the helix-helix interface, and/or serve as part of the structural unit upon reorganization by the TCRα arginine (Call et al, 2006). It has been speculated that the acidic pair may be in a state of partial ionisation to help stabilise the dimerization interface. Solution NMR studies of DAP12 also revealed a left-handed parallel dimer, though the aspartate occupies a different position in the membrane to that of CD3ζζ, whilst the stabilizing role proposed for water in the latter may instead be played by interfacial threonine hydroxyls in DAP12 (Call et al, 2010). The D/T pair contributes to a complex electrostatic network interacting with a key lysine in the NKG2C TM helix at the core of the heterotrimer, though surprisingly, one aspartate sidechain in the micelle NMR structure faced away from this network, apparently exposed to the hydrophobic lipid tail environment (Call et al, 2010). A subsequent crystallographic study in lipidic cubic phase membranes revealed the structures of DAP12 TM homotrimers and homotetramers, suggesting that the polar core of these (and perhaps other such complexes) may be further stabilized by coordinated cations, water, and/or deprotonation of the core acidic residues (Knoblich et al, 2015). Additionally, NMR structures of the anionic lipid-bound cytoplasmic domains of CD3ε (Xu et al, 2008) and the costimulatory receptor CD28 (Yang et al, 2017) revealed largely unstructured peptides, but with partially folded helical turns centered around the ITAM tyrosines. Measurement of NOEs with lipids indicated that each peptide lies at the interface between acyl tails and headgroups, with the ITAM tyrosines inserted into the hydrophobic core.

Despite the recent exciting advances in our structural knowledge regarding the nature of membrane-associated immunoreceptor assemblies, several questions remain. Given the strict
requirement for conserved ionisable residues to drive formation of the core of these complexes, it is not clear how TM domains stably insert within the hydrophobic membrane environment during early stages of assembly, which would be expected to be energetically unfavourable. Likewise, it is not clear what state of ionisation these key charged residues adopt, or whether they interact with one another via water/ions as a part of the complete complex. In addition, the nature of ITAM chains when bound to physiological membranes remains a matter of controversy (Sigalov & Hendricks, 2009; Sigalov et al, 2006), and cytoplasmic tails may exist in a dynamic equilibrium between peripherally bound and membrane-inserted states (Zimmermann et al, 2017). The MD simulation approach represents a powerful means of interrogating the nature and dynamics of such membrane-bound assemblies at unparalleled spatio-temporal resolution, and numerous simulations of components of immunoreceptor complexes have been reported. Several groups have studied the spontaneous assembly of TM components, including DAP12 and CD3εδ dimerization, and heterotrimerization of DAP12-NKG2C and TCRα-CD3εδ complexes (Wei et al, 2013; Wei et al, 2014; Sharma & Juffer, 2013). These were largely rooted in the use of coarse-grained methods to predict assembled structures for subsequent refinement in atomic detail, thereby potentially limiting their accuracy, particularly regarding specific sidechain-sidechain interactions and the representation of electrostatics within the low dielectric membrane environment. Other works have focused on atomistic refinement of the CD3ζζ homodimer (Sharma et al, 2014; Petruk et al, 2013) and DAP12-NKG2C heterotrimer (Sun et al, 2013). Recent studies by Im, Call, and co-workers demonstrated the power of combining NMR data with simulations (Krshnan et al, 2016; Park et al, 2018), for determining key interfacial sidechain conformations within the biologically relevant membrane phase (Cheng and Im, 2012). In addition, simulations of the cytoplasmic tail of CD3ε at the membrane surface revealed that it interacted more deeply with anionic phosphatidyl-glycerol lipids over
zwitterionic phosphatidyl-choline lipid bilayers, and binding was sensitive to membrane domains (Lopez et al, 2015).

Nevertheless, many of the immunoreceptor simulations reported to date were performed over relatively short timescales, and have been based on a variety of different MD forcefields, some of which may underestimate the interactions between charged amino acids (de Jong et al, 2012) that are critical to immunoreceptor complex stability. Motivated by this, we now report a comprehensive, atomic-resolution study based on extended simulation sampling (>16 µs) of several TM and cytoplasmic immunoreceptor components. We have studied membrane-bound monomeric (TCRα, TCRβ, CD3δ, CD3ε, CD3γ, CD3ζ), dimeric (CD3ζζ, DAP122), and trimeric (TCRα-ζζ, TCRα-CD3εδ, TCRβ-CD3γε, DAP122-NKG2C) systems, in order to systematically assess the dynamics of these TM assemblies in the context of a single forcefield. We also report simulations of the ITAM-containing CD3ε cytoplasmic domain (CD3εCD) embedded at various depths within membranes, and study its dependence upon alternative lipid compositions and phosphorylation states. Particular attention is paid to the effects of local microenvironment upon the stability of the various systems, helping us to identify possible determinants of functional regulation of immunoreceptor oligomers, and more generally, the architecture of multi-subunit, membrane-bound complexes.

**Methods**

**System preparation**

The sequences used in this study are shown in Table S1. The coordinates for the CD3εCD tail and the TM assemblies of CD3ζ, DAP12, and NKG2C, were based on their available NMR structures, deposited in the protein data bank (PDB codes 2K4F, 2HAC, 2L34, 2L35, respectively) (Call et al, 2006; Call et al, 2010; Xu et al, 2008). As detailed in Table S1, for
TM peptides without available experimental structures, the results of various TM structure
prediction servers including MEMSAT2 (Jones et al, 1994), TMHMM (Sonhammer et al, 1998), and TMPRED (Hofmann & Stoffel, 1993) within the GeneSilico server (Kurowski & Bujnicki, 2003) were combined with those from the literature, and used to generate a consensus. The consensus was used to select the likely regions of TM α-helicity secondary structure, which was subsequently enforced within Modeller (Sali & Blundell, 1993) to generate 50 models per sequence. The model with the lowest objective score was chosen, and further validated to ensure there were no unreasonable geometries using MolProbity4 (Chen et al, 2010) and PROCHECK (Laskowski, 1996). The initial structures of the trimeric TM complexes TCRα-CD3ζζ, TCRα-CD3εδ, and TCRβ-CD3εγ were generated by aligning each chain based on the DAP12-NKG2C structure, followed by orientation along the z-axis, ensuring the key charged residues were in close proximity to one another. To facilitate comparison of simulation systems, throughout the manuscript the residue numbering of each chain begins from 1. All ionisable residues were treated in their default charged state, except where stated otherwise. An appropriate inter-helix disulfide bridge was modelled as observed in the NMR structures of the CD3ζζ and DAP12 homodimers. A flexible linker between DAP12 and NKG2C was included in the corresponding trimer to replicate the conditions of the NMR study (Call et al, 2010).

Each TM assembly was placed in a cubic box with a minimum space of 1 nm between protein and the box edges. After an initial 1,000 steps of steepest descent energy minimization, for all simulations, the g_memb code (Wolf et al, 2010) within GROMACS was used to place the protein TM domains within a pre-equilibrated palmitoyl-oleoyl phosphatidylcholine (POPC) bilayer, such that the helices lay at the centre of the membrane in a transmembrane orientation. After embedding the protein in the membrane, a further
steepest descent energy minimization and short equilibrium run was used to relax the membrane. The system was then solvated by superposition of a box of pre-equilibrated water molecules. All the solvent molecules within 0.5 nm from a non-hydrogen atom of the protein or lipid were removed. Sodium and chloride ions were then added to bulk solvent to neutralise any net charge present in the system, to a total salt concentration of 0.1 M, to mimic physiological salt conditions. Subsequently, steepest descent energy minimization of the solvated, electroneutral system was performed (<5,000 steps) in order to ensure that the system had no steric clashes or inappropriate geometries. The system was then equilibrated to allow the solvent and lipids to relax around the protein, by applying position restraints (1,000 kJ mol\(^{-1}\) nm\(^{-2}\)) to all non-hydrogen atoms of the protein for 5 ns. This was followed by 100 to 500 ns of unrestrained production MD. For each system, three replica trajectories were generated based on different initial velocities, to improve conformational sampling.

**Alchemical membrane insertion of CD3εCD peptides**

The CD3εCD peptide was simulated at an angle perpendicular to the membrane normal, positioned at three different locations: (i) on the surface; (ii) at the headgroup/tail interface; and (iii) in the centre of a bilayer. This was performed for the peptide with the ITAM tyrosines dephosphorylated and phosphorylated, for both pure POPC membranes and for mixed membranes containing POPC and palmitoyl-oleoyl phosphatidylserine (POPS) in an 88:12 ratio, thus yielding a total of twelve systems. In the mixed membrane systems, the POPS lipids were asymmetrically distributed in the “cytoplasmic” leaflet, except for the systems in which the peptide was in the center of the bilayer, where the POPS was evenly distributed in both leaflets to ensure sufficient protein-lipid interactions were observed. The asymmetric mixed lipid bilayer was built using the CHARMM membrane builder (Jo et al, 2009). An alchemical method based on free energy perturbation (FEP) was used to slowly
“grow” the CD3εCD peptide as a function of the coupling parameter $\lambda$, at the three different locations in the bilayer. For each system, we first gradually switched on the van der Waals interactions, from $\lambda_{vdw}=0$ to $\lambda_{vdw}=1$, and subsequently gradually switched on the electrostatic interactions (from $\lambda_{coul}=0$ to $\lambda_{coul}=1$). In each coupling run, 21 $\lambda$ windows were used, i.e. $\lambda=0, 0.05, 0.1, \ldots, 0.9, 0.95, 1.0$. The length of each window simulation was 0.5 ns, and the total duration for introducing each peptide into the bilayer was therefore 21 ns. For each value of $\lambda$, a complete workflow (entailing steepest descents minimisation, L-BFGS minimisation, $NVT$ equilibration, $NPT$ equilibration, and data collection) was conducted. The protein was position restrained (force constant of 1000 kJ mol$^{-1}$ nm$^{-2}$ applied to all non-hydrogen atoms) during this process and then removed later after being fully coupled (i.e. at $\lambda_{vdw}=1$, $\lambda_{coul}=1$), during subsequent 5 ns equilibration and 500 ns production runs.

**Simulation protocol**

All simulations were carried out with the GROMACS package version 4.5.5 (Berk et al, 2008), using the CHARMM27/CMAP all-atom forcefield (Brooks et al, 2009) for the protein and CHARMM36 for lipids (Klauda et al, 2010), with TIP3P water (Jorgensen, 1983; Bjelkmar, 2010). Phosphotyrosine parameters for the ITAM motifs were taken from previous work (Bond & Faraldo-Gómez, 2011). All atomistic simulations were performed in the $NPT$ ensemble, at a pressure of 1 atm and temperature of 310 K, above the gel to liquid-crystalline phase transition temperatures for POPC (271 K) or POPS (300 K). Temperature coupling used the velocity-rescaling thermostat (Bussi et al, 2007) and a coupling time of 1.0 ps. Semi isotropic pressure coupling used the Parrinello-Rahman barostat (Parrinello & Rahman, 1981) with a coupling constant of 5 ps. The leap-frog algorithm with a 2 fs time step was employed to integrate the equations of motion. The LINCS algorithm was used to constrain bonds connected to hydrogens (Hess et al, 1997). Non-bonded pair lists were generated every 10
steps with a distance cut-off of 1.2 nm. The van der Waals interactions were smoothly switched off between 1.0 and 1.2 nm. A cut-off of 1.4 nm was used, with the potential shift Verlet cut-off scheme. Pair lists were updated every 10 steps. Long-range electrostatic interactions were treated using the Particle-Mesh-Ewald algorithm, with a real-space sum cut-off of 1.2 nm (Essman et al, 1995). All simulations were carried out under periodic boundary conditions. Molecular graphics analyses were performed using the VMD package (Humphrey et al, 1996).

Results and Discussion

Dynamics of a signaling module tail at different membrane locations

It is at present unclear precisely where the cytoplasmic tails of signaling domains such as CD3εCD partition within the biologically relevant membrane environment, or how changes in the composition of immunoreceptor associated lipid microdomains regulate ITAM tyrosine exposure to phosphorylation at the molecular level. We used alchemical simulation methods to slowly “grow” the dephosphorylated (ITAM) and phosphorylated (pITAM) states of the 57-residue CD3εCD tail at different positions within both a pure POPC bilayer and an asymmetric one containing ~12% of POPS on the “cytoplasmic” side. Phosphatidylserine is the most abundant anionic lipid in vivo, localizes to the cytoplasmic leaflet (Devaux, 1991), and is thought to play important regulatory roles in TCR activation within local rafts (Gagnon et al, 2012). The CD3εCD peptides were embedded at three locations into each lipid bilayer, including: (i) in the middle of the membrane, between both leaflets (ITAM\textsubscript{PC\_center}, ITAM\textsubscript{PC/PS\_center}, pITAM\textsubscript{PC\_center}, pITAM\textsubscript{PC/PS\_center}); (ii) at the interface of the cytoplasmic leaflet between lipid tails and headgroups (ITAM\textsubscript{PC\_interface}, ITAM\textsubscript{PC/PS\_interface}, pITAM\textsubscript{PC\_interface}, pITAM\textsubscript{PC/PS\_interface}); and (iii) on the surface of the cytoplasmic leaflet, above the headgroups (ITAM\textsubscript{PC\_surface}, ITAM\textsubscript{PC/PS\_surface}, pITAM\textsubscript{PC\_surface}, pITAM\textsubscript{PC/PS\_surface}).
Following membrane insertion of each peptide, a 500 ns production simulation was performed to equilibrate its interactions with the local environment. The CD3εCD tail consists of a juxtamembrane N-terminal polybasic region containing 10 basic residues (residues 1-29) and a C-terminal region containing two ITAM motifs, separated by a central polyproline region (residues 30-36) known to bind the adaptor protein Nck (Gil et al, 2002).

Figure 1 shows the final simulation snapshot of each system, highlighting the interaction of the basic residues and (phospho)tyrosines with the surrounding lipids. Visual analysis revealed different degrees of distortion of the membrane, which contributes to the stability of the CD3εCD systems. A very distorted membrane with lipid headgroups (and solvent) entering the core of the membrane from both sides in the ITAM<sub>PC_center</sub>, ITAM<sub>PC/PS_center</sub>, pITAM<sub>PC_center</sub>, and pITAM<sub>PC/PS_center</sub> systems suggests that CD3εCD is in a "non-preferred" position (Figure 1A, 1D, 1G, 1J). This results from the presence of multiple arginine and lysine residues within the low dielectric acyl tails region, and consequently partitioning here would be highly unfavored, irrespective of the lipid composition or phosphorylation status of the ITAM tyrosines. Thus, further discussion of these systems is omitted.

The systems containing CD3εCD at the interfacial region exhibited less membrane disruption. Most basic residues interacted with the headgroup phosphates and/or phosphatidyl-serine groups at the cytoplasmic interface, though one or two typically exhibited “snorkelling” to the opposing leaflet, thereby leading to some local lipid deformation and membrane thinning (Figure 1B, 1E, 1H, 1K). For the fully surface bound systems, whilst several basic residues were bound to the lipid headgroups, many others were exposed to solvent, and as a result, local lipid deformation was absent (Figure 1C, 1F, 1I, 1L). In some cases the N-terminal polybasic and proline-rich stretches were observed to detach from the membrane, helping the
ITAM to remain in the “cytoplasm”. This may arise from the inherent disorder in these regions of the peptide, evident from the erratic pattern of conformational dynamics, as assessed via their backbone Ca root-mean-square deviations (RMSDs) relative to the NMR structure (Table 1), and by the diverse mixture of interchanging secondary structures across the N-terminal region (Figure S1).

For the interfacial systems, the RMSD for both the CD3εCD tail and for the isolated C-terminal dual ITAM region increased from ~0.3 nm to ~0.4 nm upon phosphorylation (Table 1). Concomitantly, the solvent accessible surface area (SASA) of ITAM tyrosines Y38 and Y49 tended to increase, becoming less deeply embedded within the hydrophobic core of the membrane (Table 1). While the regions around both ITAM motifs tended to form intermittent turn and 3_10-helical structures across all systems, the switch from mixed PC/PS lipids to a purely zwitterionic PC environment led to the loss of canonical α-helices centered around the second ITAM (Figure S1). The RMSDs for the entire peptide were significantly higher across surface-bound systems compared to the interfacial ones, ranging from ~0.5-0.8 nm, in agreement with their increased dynamics and weaker membrane attachment (Figure 1). In contrast with the pattern observed for interfacial systems, the RMSDs for the isolated ITAM segment of the surface-bound tails were higher in the unphosphorylated (~0.7-1.0 nm) versus phosphorylated (~0.5 nm) states, accompanied by almost complete exposure of Y38 and Y49 to solvent (Table 1). Phosphorylation fully stabilized several α-helical turns around Y49, irrespective of the lipid microenvironment.

Taken together, our results suggest that the CD3εCD tail may stably exist near to the bilayer interface in both fully membrane-inserted or peripherally bound states, consistent with recent surface plasmon resonance and neutron reflection measurements of such an equilibrium
The conformation of the peptide was found to be sensitive to its local microenvironment, particularly in the case of the second ITAM motif centered around Y49. Interestingly, NOE measurements suggested that this tyrosine is less deeply buried than Y38 in the resting state (Xu et al, 2008). In our simulations, removal of POPS lipids around CD3εCD at the cytoplasmic interface resulted in a loss of α-helical structure around Y49, whereas its phosphorylation at the membrane surface over-stabilized the α-helix and exposed the ITAMs to solvent. Thus, the Y49 region in particular may be important in governing the switch between interfacial and surface-bound configurations; upon T-cell stimulation, the loss of anionic lipid rafts stabilize helical states that favour partitioning to the membrane surface, thus exposing the ITAMs to cytoplasmic kinases. Tyrosine phosphorylation then helps to maintain the structured CD3εCD tails, ensuring amplification of downstream signaling cascades.

**Dynamics of monomeric immunoreceptor transmembrane domains**

Each immunoreceptor TM domain contains at least one ionizable residue proposed to be involved in pairwise interactions during receptor assembly (Wucherpfennig et al, 2009). It is presently not clear how such domains are stably expressed in the membrane in their pre-assembled state, given the energetic barrier for insertion of charges within the low dielectric acyl tail phase. To investigate this, we therefore carried out simulations of the individual, isolated α-helices corresponding to the TCRα, TCRβ, CD3δ, CD3ε, CD3γ, and CD3ζ TM sequences. Each peptide was embedded in a TM orientation within a POPC lipid bilayer, and a series of 100 ns trajectories were generated, performed in triplicate to increase conformational sampling.
The conformations of all monomers remained relatively stable over the simulation timescale, with RMSDs for the backbone Cα atoms reaching on average ~0.2-0.3 nm with respect to the respective initial structure (Table 2). Correspondingly, little loss of α-helicity was observed outside of the peptide termini (Figure S2), in spite of the presence of ionizable residues within the TM region. On the other hand, the charged TM domains exhibited significant changes in the context of their interactions with the local environment as the simulations progressed. Thus, from their initially perpendicular positions, each helix adopted increasingly tilted orientations with respect to the bilayer normal, ranging from between ~20-50° across systems (Table 2).

Visual inspection suggests that such behaviour was driven by the tendency for the ionisable residues to attain more favourable configurations with respect to the local environment (Figure 2). The least tilted helix was TCRβ, which contains a single charged arginine in its TM region that could “snorkel” to the polar lipid bilayer interface, whilst the remaining CD3 peptides exhibited more significant degrees of tilting due to the lack of capacity for “snorkelling” of the single acidic residue found in each of their TM regions. The separated pair of arginine and lysine residues distributed along TCRα resulted in the most tilted helix of all systems. Consistently, the bilayer lipids were locally deformed (Figure 2), in all cases leading to electrostatically driven binding of POPC phosphate or choline groups with the basic or acidic TM sidechains, respectively (Table 2). This was accompanied by nearby water molecules and, in some of the CD3 systems, sodium counterions (Table 2). Collectively, these results suggest that the ionisable residues of single immunoreceptors may be stably embedded within the membrane phase via a combination of helix tilting and lipid membrane deformation, ensuring their solvation and at least partial electroneutralization.
Dynamics of dimeric immunoreceptor assemblies in different charge configurations

The solution NMR structures of DAP12-DAP12 and CD3ζζ within detergent micelles revealed several related features, including a pair of crucial aspartates at the core of a left-handed α-helical bundle (Call et al, 2006; Call et al, 2010). It is unclear how such assemblies may be stably accommodated within the low dielectric lipid tail environment, or how their associated like-charge residues stably exist in proximity to one another, so as to form the necessary functional interface for productive higher-order oligomerization. A series of triplicate 500 ns simulations of the DAP12-DAP12 and CD3ζζ homodimers were thus next performed to probe these questions, and in particular, to investigate previously hypothesized roles for stabilization of the membrane-embedded acidic groups by partial deprotonation, solvation, or complexation by coordinated cations (Call et al, 2006; Call et al, 2010; Knoblich et al, 2015). A total of four membrane-embedded systems were investigated, in which each dimer was fully or only partially deprotonated on the acidic pair, i.e. in CD3ζζ both (CD3ζζ) or only one (CD3ζζ0ζζ) of two D9 carboxylates were charged, and likewise, in DAP12-DAP12 both (DAP12·DAP12−) or only one (DAP120·DAP12−) of two D16 carboxylates were charged.

The magnitude of Ca RMSDs, both for the individual chains and for the dimeric complexes, were in general higher for the fully charged systems compared to their partially charged counterparts (Table 3). This was consistent with the tendency for the N-terminal halves of the TM cores in CD3ζζ and DAP12·DAP12− to exhibit intermittently unstable α-helicity (Figure S3), and for some concomitant bending/kinking of these helices to occur (Figure 3A, B). Nevertheless, compared to their respective NMR structures, the buried area between chains was either maintained, at ~4 nm² for DAP12-DAP12, or significantly increased from ~6 to ~10 nm² for CD3ζζ, suggesting that the increased lateral pressure of a lipid bilayer compared to the micelle environment may help to drive homodimer assembly. The helix-helix crossing
angles of the dimeric complexes were not significantly different from their corresponding NMR structures, but like their monomeric counterparts, the individual helices adopted tilted angles with respect to the bilayer normal of between ~10-30° (Table 3, Figure 3A, B).

The pairs of aspartate sidechain carboxylates remained in close proximity to one another for both peptides, irrespective of ionization state (Table 3), though the separation was on average ~0.1 nm less for the partially deprotonated systems, consistent with the stable hydrogen-bonds observed between acidic groups throughout the CD3ζζ and DAP12ζζDAP12ζζ trajectories (Figure 3C, 3E). Additional stabilizing contacts identified by structural and mutational analyses (Call et al, 2006; Call et al, 2010) also helped to maintain each interface. In CD3ζζ, inter-chain hydrogen bonds were consistently observed between the hydroxyl groups of Y15 and T20, with the latter’s orientation also stabilized by intra-chain carbonyl hydrogen-bonds (Table 3, Figure 3D). Likewise, in DAP12-DAP12, intra-chain hydrogen bonds were consistently observed between the hydroxyl groups of the crucial T20 residue and the backbone carbonyl oxygen of D16 (Table 3, Figure 3E). Overall, the average TM interfaces were well conserved irrespective of ionization state (Figure S4), with further hydrophobic inter-chain contacts formed by the sidechains of L12, L19, V26 and T20 in CD3ζζ, and by L9, I12, V13, V19 and T20 in DAP12-DAP12.

We sought to understand the stability of the acidic cores even in their fully deprotonated states. In contrast with the monomeric systems, the local bilayer structure remained relatively unperturbed around the more effectively “shielding” dimeric assemblies. Nevertheless, single POPC choline groups in the upper bilayer leaflet were able to bind and help neutralize D9 in CD3ζζ, facilitated in some cases by nearby sodium ions (Figure 3C). On the other hand, lipid headgroups did not significantly interact with the aspartate pair of DAP12ζζDAP12ζζ,
likely due to the more deeply buried location of D16, positioned around the center of the TM domain (Figure 3B). Instead, sodium ions were consistently bound (Table 3), cross-linking the aspartate carboxylates (irrespective of charge), facilitated in some cases by T20 sidechain hydroxys, along with water molecules (Figure 3E). Interestingly, Jusoh et al used simulations to show that local microsolvation could stabilize pairs of centrally located charged or polar amino acids in TM helix dimers (Jusoh & Helms, 2011).

Thus, whilst the partially deprotonated forms of each dimer system appear to be marginally more stable, the fully deprotonated states are not precluded from membrane insertion, thanks to electroneutralizing interactions with either lipid headgroups or counterions, in CD3ζζ or DAP12-DAP12, respectively. The cross-linking of the DAP12 homodimers observed here lends further support to recent crystallographic data indicating a structural role for cations in binding to the central acidic core of DAP12 homotrimers and homotetramers (Knoblich et al, 2015). Furthermore, the presence of clusters of water molecules, apparent across all simulations, proximal to the acidic pairs (Table 3, Figure 3C, 3E) suggests that their state of ionization may be dynamic, enabling the TM domains to cycle between conformational equilibria favouring dimeric versus high-order immunocomplexes.

**Dynamics of intact trimeric immunoreceptor complexes within the membrane**

The NMR structure of the DAP12-NKG2C-DAP12 heterotrimer suggested that the D16/T20 pair in each DAP12 TM domain forms the interaction site for K19 in NKG2C, as part of a 5-residue “assembly motif” (Call et al, 2010). Puzzlingly, however, only one of the two aspartates was observed to be in direct contact with the lysine, with the other apparently facing the hydrophobic lipid environment. Thus, to examine the stability of this TM charge-network, triplicate 500 ns simulations of the membrane-embedded DAP12-NKG2C-DAP12
complex were performed. For comparison, and to assess how generally applicable the 5-
residue motif may be to other immunoreceptor complexes, triplicate 100 ns trajectories were
also generated for models of each of the TCRα-CD3ζζ, TCRα-CD3εδ, and TCRβ-CD3εγ
heterotrimers.

The stability of each complex was relatively similar, with Cα RMSDs for individual helices
or the entire assemblies reaching 0.3-0.4 nm, relative to the respective NMR structure or
starting model (Table 4). Like the dimeric systems, conformational changes were limited to
the N-terminal ends of the TM domains, where some bending/kinking and intermittent loss of
α-helicity was evident (Figures 4, S5). Some tendency for tilting of TM helices was also
evident (Table 4), though less pronounced than in the case of the homodimeric simulations.
Crossing angles were similarly reduced in comparison with the homodimeric systems, and for
DAP12-NKG2C-DAP12, did not significantly differ for the simulation versus NMR data.

The TM helices of the DAP12-NKG2C-DAP12 complex remained closely packed over each
500 ns trajectory. Whilst the homodimeric DAP12 interface typically exhibited a loss of ~1
nm² of buried surface area compared to the NMR structure, this was compensated by a total
increase of ~2 nm² in buried area at the heterodimeric interfaces. These were asymmetric in
the NMR structure, with buried areas of 5.1-7.6 nm², but reached 7.2-7.3 nm² at both sites
during simulation, concomitant with the adjustment of the trimeric core such that both D16
carboxylates on DAP12 reoriented (facilitated by intra-chain hydrogen bonds with T20
hydroxyls) to stably interact with K19 on NKG2C (Figure 4A). The inter-helical buried
surface areas were more erratic for the remaining TCR-CD3 heterotrimer models, due to the
*ab initio* predicted nature of their interfaces. Nevertheless, the corresponding crucial charge-
charge interaction sites were reproduced across systems (Figure 4B-D), supported by
sidechain hydroxyls of a nearby serine or threonine on the CD3 chains equivalent to T20 of
DAP12, whose mutation has been shown to cause assembly defects (Call et al, 2010). Whilst
water was observed to partially hydrate this polar core across all systems, lipid headgroups or
ions were no longer required for electroneutralization, in contrast with the monomeric or
dimeric systems. The exception to this was the TCRβ-CD3εγ complex, in which a sodium ion
was found coordinated to the CD3γ E14 carboxylate in two out of three replicas (Figure 4C).
Interestingly, this is the only one of the TM domain cores in which the longer glutamate is
found instead of aspartate, and is also the only case in which the hydroxyl-bearing residue
(CD3γ S17) lies at the i+3 instead of i+4 position relative to the acidic site. This reduces the
capacity for additional intra-chain hydrogen bonding, consistent with evidence that TCRβ
associates less strongly with CD3εγ than TCRα does with CD3εδ (Call et al, 2002). In
summary, our data support a symmetrical 5-residue “assembly motif” as the fundamental
structural unit of immunoreceptor complexes, which may have been evolutionarily modulated
by subtle sequence variations to fine-tune complex stabilities / lifetimes.

Conclusions
In this study, we have explored molecular aspects of the interactions between the subunits of
immunoreceptor complexes and the membrane, and their potential role in regulating early
events that follow TCR stimulation. The precise mechanisms by which activation is
transduced to cytoplasmic signaling modules are unclear, but our extended simulations of the
CD3εCD tail confirm that its conformation is highly sensitive to its local microenvironment,
comprising the ITAM regions themselves, whose structural stability and solvent exposure
were governed both by the membrane lipid composition and phosphorylation. Furthermore,
whilst it is well established that TM immunoreceptor complexes are formed by the interaction
between conserved ionizable residues (Call et al, 2006; Call et al, 2010; Knoblich et al,
2015), this is at odds with the large energetic penalty associated with transfer of charges into the low dielectric hydrophobic core of lipid bilayers (MacCallum et al, 2007). Based on our data, we predict that in the early stages of assembly, membrane insertion of each TM domain is facilitated by optimization of its interactions with constituents of the local membrane interface environment, including lipid headgroups, solvent water, or counterions, supporting other computational (Sharma et al, 2014; Petruk et al, 2013; Cheng and Im, 2012) and structural studies (Call et al, 2006; Knoblich et al, 2015). In addition, particularly in the dimeric forms of signaling modules, a state of partial ionization of the central acidic pair seems likely. Such a model for the stable insertion of charge-pairs may be a general solution to the satisfaction of exposed charges in the association of TM complexes (Senes et al, 2004). The non-covalent association of such homodimeric TM assemblies may only be meta-stable, but would likely be sufficient to expose pairs of nearby N-terminal cysteines (as in TCRαβ, DAP12, or CD3ζζ) to the lumen of the ER for inter-helical disulfide bond formation. Finally, our data and those of others (Wei et al, 2014; Sharma & Juffer, 2013; Sun et al, 2013; Cheng and Im, 2012) support the concept of a symmetric interaction core arranged around a conserved network of ionizable and nearby polar residues in heterotrimeric assemblies, thus defining a fundamental building block in the architecture of diverse immunoreceptor complexes.

Acknowledgments

We acknowledge access to the Darwin supercomputer of the University of Cambridge, and the HECToR UK supercomputer service for computational resources awarded by CCP-BioSim. ND thanks Maite Ortiz-Suarez and Mark Williamson for assistance during simulation analysis, and the Nehru Trust of the University of Cambridge and Rajiv Gandhi (UK) foundation for financial support.
Table 1. Conformational dynamics and ITAM tyrosine exposure of CD3ε:CD ITAM systems. The values were calculated as the average over the last 100 ns of each 500 ns simulation.

<table>
<thead>
<tr>
<th></th>
<th>Ca RMSD, whole peptide (nm)</th>
<th>Ca RMSD, basic N-terminus, residues 1-29 (nm)</th>
<th>Ca RMSD, proline-rich region, residues 30-36 (nm)</th>
<th>Ca RMSD, ITAM region, residues 37-56</th>
<th>SASA, Y38 (nm²)</th>
<th>SASA, Y49 (nm²)</th>
</tr>
</thead>
</table>
Table 2. Dynamics and interactions of monomeric systems including TCRα, TCRβ, CD3δ, CD3ε, CD3γ and CD3ζ. The mean and SD were obtained from the final 10 ns of triplicate 100 ns trajectories for each system. Cα RMSD values were calculated after excluding the flexible N- and C-termini.

<table>
<thead>
<tr>
<th></th>
<th>TCRα</th>
<th>TCRβ</th>
<th>CD3δ</th>
<th>CD3ε</th>
<th>CD3γ</th>
<th>CD3ζ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cα RMSD (nm)</td>
<td>0.3 ± 0.05</td>
<td>0.2 ± 0.05</td>
<td>0.3 ± 0.08</td>
<td>0.3 ± 0.06</td>
<td>0.3 ± 0.09</td>
<td>0.3 ± 0.06</td>
</tr>
<tr>
<td>Helix tilt angle (°)</td>
<td>49 ± 5</td>
<td>20 ± 10</td>
<td>37 ± 6</td>
<td>28 ± 4</td>
<td>34 ± 12</td>
<td>31 ± 4</td>
</tr>
<tr>
<td>Minimum distance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>between POPC headgroup</td>
<td>Phosphate/R13 0.2 ± 0.1</td>
<td>Phosphate/K13 0.2 ± 0.1</td>
<td>Choline/D14 0.3 ± 0.1</td>
<td>Choline/D14 0.5 ± 0.1</td>
<td>Choline/E14 0.4 ± 0.2</td>
<td>Choline/D9 0.5 ± 0.2</td>
</tr>
<tr>
<td>and key ionizable</td>
<td>Phosphate/K18 0.3 ± 0.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>residue sidechains (nm)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Minimum distance</td>
<td>Chloride/R13 1.6 ± 0.4</td>
<td>Chloride/K13 1.8 ± 0.4</td>
<td>Sodium/D14 1.0 ± 0.8</td>
<td>Sodium/D14 0.5 ± 0.6</td>
<td>Sodium/E14 0.2 ± 0.1</td>
<td>Sodium/D9 0.7 ± 0.7</td>
</tr>
<tr>
<td>between ions &amp;</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>key ionisable residue</td>
<td>Water/R13 0.2 ± 0.1</td>
<td>Water/K13 0.2 ± 0.1</td>
<td>Water/D14 0.2 ± 0.1</td>
<td>Water/D14 0.2 ± 0.1</td>
<td>Water/E14 0.2 ± 0.1</td>
<td>Water/D9 0.2 ± 0.1</td>
</tr>
<tr>
<td>sidechains (nm)</td>
<td>Water/K18 0.2 ± 0.1</td>
<td></td>
<td></td>
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Table 3. Dynamics and interactions of dimeric systems including CD3ζζ, CD3ζθζ, DAP12-DAP12' and DAP12θ-DAP12'. The mean and SD were obtained from the final 100 ns of triplicate 500 ns trajectories for each system. Cα RMSD values were calculated after excluding the flexible N- and C-termini.

<table>
<thead>
<tr>
<th></th>
<th>CD3ζζ</th>
<th>CD3ζθζ</th>
<th>DAP12-DAP12'</th>
<th>DAP12θ-DAP12'</th>
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<tbody>
<tr>
<td>Cα RMSD (nm)</td>
<td>Both chains</td>
<td>Both chains</td>
<td>Both chains</td>
<td>Both chains</td>
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<td>Both chains</td>
<td>Both chains</td>
<td>Both chains</td>
<td>Both chains</td>
</tr>
<tr>
<td></td>
<td>0.3 ± 0.03</td>
<td>0.2 ± 0.03</td>
<td>0.5 ± 0.08</td>
<td>0.4 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.3 ± 0.05</td>
<td>0.2 ± 0.02</td>
<td>0.5 ± 0.10</td>
<td>0.3 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>0.3 ± 0.03</td>
<td>0.3 ± 0.03</td>
<td>0.5 ± 0.07</td>
<td>0.4 ± 0.04</td>
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<tr>
<td>Helix tilt angle (°)</td>
<td>CD3ζζ</td>
<td>CD3ζθζ</td>
<td>DAP12-DAP12'</td>
<td>DAP12θ-DAP12'</td>
</tr>
<tr>
<td></td>
<td>18 ± 7</td>
<td>20 ± 7</td>
<td>33 ± 9</td>
<td>27 ± 6</td>
</tr>
<tr>
<td></td>
<td>10 ± 5</td>
<td>34 ± 8</td>
<td>23 ± 7</td>
<td>33 ± 9</td>
</tr>
<tr>
<td>Helix-helix</td>
<td>CD3ζζ</td>
<td>CD3ζθζ</td>
<td>DAP12-DAP12'</td>
<td>DAP12θ-DAP12'</td>
</tr>
<tr>
<td></td>
<td>22 ± 4</td>
<td>25 ± 3</td>
<td>27 ± 12</td>
<td>16 ± 4</td>
</tr>
<tr>
<td></td>
<td>(NMR structure = 23)</td>
<td>(NMR structure = 23)</td>
<td>(NMR structure = 20)</td>
<td>(NMR structure = 20)</td>
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<td>0.3 ± 0.03</td>
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<tr>
<td>between key acidic</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>residue sidechains</td>
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<td></td>
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<tr>
<td>Additional inter-/intra-chain residue minimum distances (nm)</td>
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<tr>
<td>Y15/T20</td>
<td>0.2 ± 0.02</td>
<td>0.4 ± 0.3</td>
<td>0.2 ± 0.03</td>
<td>0.2 ± 0.01</td>
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<tr>
<td>Y15'/T20</td>
<td>0.2 ± 0.04</td>
<td>0.2 ± 0.02</td>
<td>0.2 ± 0.03</td>
<td>0.2 ± 0.03</td>
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<tr>
<td>Minimum distance</td>
<td>Choline/D9</td>
<td>Choline/D9</td>
<td>Choline/D16</td>
<td>Choline/D16</td>
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<tr>
<td>between POPC headgroup &amp; key ionic residue sidechains (nm)</td>
<td>0.4 ± 0.2</td>
<td>0.7 ± 0.3</td>
<td>0.9 ± 0.5</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Choline/D9'</td>
<td>Choline/D9'</td>
<td>Choline/D16'</td>
<td>Choline/D16'</td>
</tr>
<tr>
<td></td>
<td>0.5 ± 0.2</td>
<td>0.5 ± 0.2</td>
<td>0.8 ± 0.4</td>
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<tr>
<td>Minimum distance</td>
<td>Sodium/D9</td>
<td>Sodium/D9</td>
<td>Sodium/D16</td>
<td>Sodium/D16</td>
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<tr>
<td>between ions &amp; key ionic residue sidechains (nm)</td>
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<td>0.3 ± 0.07</td>
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<tr>
<td></td>
<td>Sodium/D9'</td>
<td>Sodium/D9'</td>
<td>Sodium/D16'</td>
<td>Sodium/D16'</td>
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<tr>
<td></td>
<td>0.7 ± 0.8</td>
<td>0.8 ± 0.8</td>
<td>0.2 ± 0.01</td>
<td>0.2 ± 0.02</td>
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<td>between water molecules &amp; key ionic residue sidechains (nm)</td>
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<td>0.3 ± 0.15</td>
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</tr>
<tr>
<td></td>
<td>Water/D9'</td>
<td>Water/D9'</td>
<td>Water/D16'</td>
<td>Water/D16'</td>
</tr>
<tr>
<td></td>
<td>0.2 ± 0.01</td>
<td>0.2 ± 0.01</td>
<td>0.1 ± 0.01</td>
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Table 4. Dynamics and interactions of trimeric systems including DAP12-NKG2C-DAP12, TCRα-CD3ζδ, TCRβ-CD3γε and TCRα-CD3ζζ. The mean and SD were obtained from the final 100 ns of triplicate 500 ns trajectories for DAP12-NKG2C-DAP12, and from the final 10 ns of triplicate 100 ns trajectories for the remaining systems. Cu RMSD values were calculated for all chains of the protein, after excluding flexible N- and C-termini.

<table>
<thead>
<tr>
<th></th>
<th>Cu RMSD (nm)</th>
<th>All chains 0.4 ± 0.04</th>
<th>All peptides 0.3 ± 0.02</th>
<th>All peptides 0.3 ± 0.2</th>
<th>All peptides 0.3 ± 0.2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DAP12-NKG2C-DAP12</td>
<td>NKG2C 0.3 ± 0.02</td>
<td>DAP12 0.3 ± 0.04</td>
<td>DAP12' 0.4 ± 0.09</td>
<td>TCRα 0.3 ± 0.08</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>Helix tilt angle (°)</td>
<td></td>
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<tr>
<td></td>
<td>DAP12-D16/DAP12'-D16</td>
<td>0.4 ± 0.04</td>
<td>CD3ε-D14/CD3α-D14</td>
<td>CD3ε-D14/CD3α-D14</td>
<td>CD3ε-D14/CD3α-D14</td>
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<tr>
<td>Minimum distance between key acidic residue sidechains (nm)</td>
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</tr>
<tr>
<td></td>
<td>DAP12-D16/NKG2C-K19</td>
<td>0.2 ± 0.01</td>
<td>CD3ε-D14/TCRα-K18</td>
<td>0.3 ± 0.09</td>
<td>CD3ε-D14/TCRα-K18</td>
</tr>
<tr>
<td></td>
<td>DAP12-D16/NKG2C-K19</td>
<td>0.2 ± 0.01</td>
<td>CD3ε-D14/TCRα-K18</td>
<td>0.2 ± 0.05</td>
<td>CD3ε-D14/TCRα-K18</td>
</tr>
<tr>
<td></td>
<td>Water/DAP12-D16</td>
<td>0.2 ± 0.01</td>
<td>Water/CD3ε-D14</td>
<td>0.2 ± 0.01</td>
<td>Water/CD3ε-D14</td>
</tr>
<tr>
<td></td>
<td>Water/DAP12-D16'</td>
<td>0.2 ± 0.01</td>
<td>Water/CD3ε-D14</td>
<td>0.2 ± 0.01</td>
<td>Water/CD3ε-D14</td>
</tr>
<tr>
<td></td>
<td>Water/NKG2C-K19</td>
<td>0.2 ± 0.03</td>
<td>Water/TCRα-K18</td>
<td>0.2 ± 0.01</td>
<td>Water/TCRα-K18</td>
</tr>
<tr>
<td></td>
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</tr>
</tbody>
</table>
**Figure 1. Visual analysis of CD3γCD ITAM simulations.** The final 500 ns simulation frame is depicted for each system including (A) ITAM\textsuperscript{PC\_center}, (B) ITAM\textsuperscript{PC\_interface}, (C) ITAM\textsuperscript{PC\_surface}, (D) ITAM\textsuperscript{PC/PS\_center}, (E) ITAM\textsuperscript{PC/PS\_interface}, (F) ITAM\textsuperscript{PC/PS\_surface}, (G) pITAM\textsuperscript{PC\_center}, (H) pITAM\textsuperscript{PC\_interface}, (I) pITAM\textsuperscript{PC\_surface}, (J) pITAM\textsuperscript{PC/PS\_center}, (K) pITAM\textsuperscript{PC/PS\_interface}, and (L) pITAM\textsuperscript{PC/PS\_surface}. The protein backbone is shown in green ribbons, with basic residues or (phospho)tyrosines in blue or red licorice format, respectively. POPC and POPS lipid headgroups are shown in CPK licorice format.
Figure 2. Visual analysis of monomeric TM systems during 100 ns simulations. Representative snapshots based on triplicate simulation trajectories are shown for (A) TCRα, (B) TCRβ, (C) CD3δ, (D) CD3ε, (E) CD3γ and (F) CD3ζ. For each system, panels from left to right show: side-view of the initial frame; side-view and cross-sectional top-view of the final frame, highlighting interactions with surrounding environment; and the complete simulation system at the final frame. The protein is shown in cartoons representation with sidechains in licorice CPK format; POPC lipids are shown in white licorice with nitrogen and phosphorus atoms indicated as small spheres colored dark blue or tan, respectively; water oxygens are indicated by small light-blue spheres; and sodium or chloride ions are indicated by large spheres colored yellow or magenta, respectively.
Figure 3. Visual analysis of dimeric TM systems during 500 ns simulations. Representative snapshots based on triplicate simulation trajectories are shown for (A) CD3ζζ and (B) DAP12-DAP12 homodimers; panels depict, from left to right, NMR structure, and final frames of simulation system for fully deprotonated and partially deprotonated TM acidic pairs. In (C), top-views highlighting helix-helix interactions around the D9 acidic pair are shown for CD3ζζζ− (left) and (D) CD3ζ0ζ− (right). In (D), side-views representative of both fully and partially deprotonated CD3ζζ are shown, highlighting inter-chain Y15/T20′ and intra-chain T20/G16 hydrogen bonds. In (E), side-views highlighting helix-helix interactions around the D16 acidic pair are shown for DAP12−-DAP12− (left) and DAP120-DAP120− (right). The protein is shown in green cartoons representation with sidechains in licorice CPK format; POPC lipids are shown in white licorice with nitrogen and phosphorus atoms indicated as small spheres colored dark blue or tan, respectively; water oxygens are indicated by small light-blue spheres; and sodium or chloride ions are indicated by large spheres colored yellow or magenta, respectively.
Figure 4. Visual analysis of trimeric TM systems during 100-500 ns triplicate simulations. In (A), representative snapshots of DAP12-NKG2C-DAP12 trimer are shown, including, from left to right: side-view of the NMR structure; a top-view at t=0 ns (top) and t=500 ns (bottom); and full system view at t=500 ns. Comparative snapshots are also shown for (B) TCRα-CD3εδ, (C) TCRβ-CD3εγ and (D) TCRα-CD3ζζ trimers; these panels depict top-view (left) and full system view (right) at t=100 ns. The protein is shown in cartoons representation (colored pink for NKG2C and TCRα/β, green for DAP12 and CD3δ/ζ, and tan for CD3ε/γ) with sidechains in licorice CPK format; POPC lipids are shown in white licorice with nitrogen and phosphorus atoms indicated as small spheres colored dark blue or tan, respectively; water oxygens are indicated by small light-blue spheres; and sodium or chloride ions are indicated by large spheres colored yellow or magenta, respectively.
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