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Title: TNFR1 transmembrane domain structure suppresses ligand-independent auto-activation but is not required for TNF-induced signaling

One-sentence summary: The specific structure and oligomeric state of the TMD is not required to support normal ligand-induced TNFR1 signaling, but native TMD self-association functions in concert with ectodomain interactions to suppress signaling in the absence of ligand.

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Abstract

TNF is a pivotal cytokine in the inflammatory response and predominantly signals through binding to TNFR1, a type I single-spanning transmembrane protein. TNFR1 is thought to exist primarily as monomers and dimers in cell membranes, and binding of trimeric ligands induces formation of signaling-competent trimers and higher-order oligomers that lead to full activation of NF- κ B and MAPK signaling pathways. Several TNF receptor superfamily

members, including TNFR1, can form specific trimeric structures through their transmembrane domains (TMDs). For Fas (CD95) and death receptor 5 (DR5, TRAILR2), this trimeric TMD structure supports ligand-induced activation, and ectodomain interactions are thought to prevent signaling in the absence of ligand. However, the functional significance of TMD interactions in TNFR1 is unknown. Here, we investigated this by replacing the native TNFR1 TMDs with natural and de novo designed ones that form stable and specific structures of defined monomeric or oligomeric states. Surprisingly, none of these TMD variants displayed defects in TNF-induced signaling, but some showed increased auto-activation in the absence of ligand. Ligand-independent activation was most prominent with a TMD sequence designed not to self associate (to form monomers). This activity was dependent on intracellular death domain interactions and exacerbated by a TRAPS-associated mutation, but was unaffected by a pre-ligand association domain (PLAD) mutation. Our results demonstrate that, unlike other TNFR family members, no specific oligomeric TMD structure is required for normal ligand-induced TNFR1 activation, but self-association through the native TMD may instead work in concert with ECD interactions to help suppress ligand-free auto-activation.

INTRODUCTION

Tumor Necrosis Factor (TNF) is a pivotal cytokine in the inflammatory response that helps organisms fight infections (1). When TNF signaling becomes dysregulated, it can drive chronic inflammatory diseases (2, 3). The importance of TNF in these diseases is demonstrated by the great success of anti-TNF drugs in treating psoriasis, rheumatoid arthritis and inflammatory bowel disease (4, 5). It has also led to intensive research into the molecular details of the signaling process for TNF and related ligands through their receptors.

49 TNF is the founding member of a large superfamily (TNFSF) of ligands and receptors. The
50 receptors are characterized by one (e.g., Fn14) to four (e.g., TNFR1) extracellular cysteine-rich
51 domains (CRDs) that are coordinated by disulfide bonds, followed by a transmembrane domain
52 (TMD) and an intracellular domain of variable size. In a subset of the TNF receptor superfamily
53 (TNFRSF) that includes TNFR1, this intracellular domain contains a death domain (DD) of
54 ~80 aa that allows the receptors to induce extrinsic apoptosis. In the unliganded state, the
55 receptors can form inactive dimers via a pre-ligand assembly domain (PLAD) located at the
56 most membrane distal part of the ectodomain (ECD) of the receptor, which is also the N-
57 terminal end of the protein (6). Binding of trimeric TNFSF ligands is believed to convert
58 receptor monomers and dimers into trimers and higher-order oligomers that activate signaling
59 (7-11). In death receptors Fas and DR5, this ligand-induced oligomerization is supported by
60 the formation of specific structures in their TMDs that may facilitate close apposition of
61 intracellular DDs for activation: Fas TMD forms trimers in model membranes (12), while DR5
62 TMD forms dimer-trimer networks (13). In both receptors, mutations that interfere with the
63 formation of these TMD structures disrupt cell death signaling, and in DR5, proteolytic
64 removal of the ectodomain spontaneously activates cell death (12, 13). The observations that
65 receptors form dimers in the absence of ligand suggests a model in which the structure of the
66 pre-ligand receptor dimer prevents TMD and DD self-associations that can subsequently form
67 when ligand is bound and are required to support activation of intracellular signaling (13). The
68 degree to which this model applies to TNF signaling through its primary receptor TNFR1 is
69 unclear. Considering that ligand binding only induces very small changes in the extracellular
70 PLAD-associated TNFR1 dimer (7, 14-16), a plausible model proposes that each half of a
71 dimer can also nucleate a trimer, establishing a lattice of TNF/TNFR (16). Because ligand
72 binding brings three TNFSFR TMDs into close apposition, it is proposed that TMD

trimerization drives a conformational change allowing the intracellular domains to oligomerize and activate signaling (17).

It is widely accepted that receptor oligomerization is crucial in signaling initiation. TNF binding to TNFR1 can initiate pro-inflammatory signaling via nuclear factor κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) pathways, as well as cell death via DD oligomerization and recruitment of TRADD/TRAF complexes (**Fig. 1**) (18-23). How TNF binding transmits activating signals through ECD/TMD interactions is incompletely understood. Pre-ligand self-association is mediated by the PLAD (amino acids 7-82 in human TNFR1) in CRD1 (6), and alanine substitution of lysine 61 (will be referred to here as K32A, according to the numbering convention that excludes the 29 amino acid signal peptide) in this domain has been reported to disrupt both pre-ligand self-association and TNF ligand binding (6), suggesting that PLAD interactions are a pre-requisite for function. CRDs 2 and 3 (aa 83-166 in human receptor) are important for ligand binding (7) and glutamine substitution of arginine 121 (R92Q) in CRD2 is associated with the disease TNF receptor-associated periodic syndrome (TRAPS) (24, 25). This mutant has been reported to exhibit constitutive activation in the absence of ligand binding in a cell viability assay (26). While the mechanism underlying this activity remains unknown, it has been proposed to alter ECD conformation or dynamics in a way that either mimics TNF binding or breaks inhibition of signaling in the pre-ligand dimer (16).

Like Fas and DR5, the human TNFR1 TMD forms a specific trimeric structure in model membranes (bicelles) (17), but whether this trimeric structure is essential for TNFR1 signaling has not been tested. We therefore sought to directly interrogate the relationship between

TNFR1 TMD structure and signaling activity. Mutations in known or suspected association motifs are often difficult to interpret because their functional effects can be due to disruption of a specific structure, formation of ill-defined alternative structures, destabilization of the receptor in the membrane, altered trafficking, or any combination of these. Furthermore, disruptive mutations only inform on the defective state(s) of the receptor, without consideration of alternative structural solutions that could achieve the same functional outcomes as the native one. To circumvent these limitations, we replaced the entire TNFR1 TMD sequence with natural and de novo designed ones that form specific monomeric, dimeric, trimeric or tetrameric TMD structures, and examined their signaling properties in the context of otherwise native TNFR1 sequences and with ECD or DD mutations. To investigate the functionality of these receptor variants, we examined several markers of TNFR1 signaling in inflammatory responses and cell death. Our results indicate that the native TMD structure functions in concert with ectodomain interactions to suppress DD aggregation-induced auto-activation in the absence of ligand, and they clearly show that no specific TMD structure or oligomeric state is absolutely required to support normal TNF-induced signaling.

RESULTS

Enforcing different oligomeric states of TNFR1 TMD does not impair TNF-induced signaling

Recently, de novo designed TMDs with defined and stable oligomeric states have been generated and used to modulate the activity of engineered receptors (27). We exploited some of these tools to alter the propensity of the TNFR1 TMD to adopt specific oligomeric states and investigate their effects on TNFR1 signaling. We replaced the naturally left-handed trimeric TNFR1 TMD (17) in full-length mouse TNFR1 with ones that form monomeric or

right-handed dimeric, trimeric and tetrameric structures (**Fig. 2A-B**). The monomeric TMD is a de novo designed sequence that does not self-associate in bacterial cell membranes (27), and the dimeric TMD is from glycophorin A (GpA), which forms a naturally right-handed dimeric structure (28, 29). The structure and oligomeric state of the de novo designed trimeric TMD has been experimentally validated through x-ray crystallography (27) and the de novo designed tetramer used here is validated by modelling and biochemical analysis (**fig. S1**).

These TNFR1 variants were cloned into a tightly regulated doxycycline (dox)-inducible lentiviral vector (30) and used to stably reconstitute *Tnfr1* deficient (*Tnfr1*^{-/-}) mouse dermal fibroblasts (MDFs). We confirmed that these TNFR1 variants were expressed in an inducible fashion and then treated cells with and without induction using soluble TNF ligand (**Fig. 2C-D**). Upon TNF binding, wild-type TNFR1 triggers degradation of the NF-κB inhibitor IκBα, phosphorylation of p65 (RelA) and also phosphorylation and activation of the MAPKs JNK1 and 2 (JNK1/2) and p38 within 30 minutes (31-33). Consistent with a tightly regulated expression of the receptors in *Tnfr1*^{-/-} cells, TNF was unable to promote NF-κB or MAPK activation in any of the variant lines in the absence of dox induction (**Fig. 2C**). Furthermore, all TNFR1 TMD variants stimulated NF-κB and MAPK activation after addition of TNF (**Fig. 2C-D**). Although there were some small differences in activation of these pathways by the different receptors, these differences likely reflect variability in the expression of the receptors rather than differences in signaling potential. Furthermore, expression of some of the receptor variants induced activation of NF-κB and MAPK even in the absence of TNF, and this was most pronounced in cells expressing the monomeric TMD (mTMD hereafter) TNFR1 variant (**Fig. 2C-D**). We also tested the ability of the variants to induce cell death stimulated by smac-mimetic. The smac-mimetic Compound A (CompA) inhibits both XIAP and cIAPs and sensitizes many cell types to TNFR1 activation-induced apoptosis (34-36). CompA alone is

not toxic to MDFs, however when combined with induction of TNFR1, whether wildtype (WT) or TMD variants, it resulted in a significant increase in cell death when compared to *Tnfr1*^{-/-} MDFs expressing GFP (**Fig. 2E**). This is consistent with the tonic activation of TNFR1 signaling induced by expression of the receptor alone. Addition of soluble TNF further increased cell death in all TNFR1 variant cell lines, and changes to the oligomeric states of the TMD had little effect (**Fig. 2E**).

Engineered monomeric TMD strongly promotes TNFR1 auto-activation

To explore ligand-independent activation in more detail, we performed an induction time course on the TNFR1 TMD variants in *Tnfr1*^{-/-} MDFs. Consistent with our previous results, NF-κB and MAPK activation was induced rapidly following receptor induction (**Fig. 3A**). When receptor expression levels were taken into account and all time points from multiple experiments were analyzed together, the auto-activation remained most prominent and consistent in the mTMD TNFR1 variant, though weaker signals were still present for trimeric and tetrameric TMD variants (**Fig. 3B**). There was also a trend towards increased cell death in cells expressing mTMD TNFR1 when co-treated with CompA (**Fig. 3C**). This suggested to us that departures from the native TMD structure and/or reduction in overall TMD self-association were responsible for increased auto-activation. As an independent test, we made single-amino-acid substitutions in the native TMD sequence that have been reported to disrupt trimerization. Specifically, human TNFR1 G221Y and L225Y reduced the TMD peptide trimers used for NMR structure determination to mostly dimers and monomers, while P215Y had no effect (37). The corresponding mouse G222Y, L226Y and P216Y mutants all expressed well and were detected at the cell surface in *Tnfr1*^{-/-} MDFs, though at slightly lower levels than WT and mTMD (**fig. S2, fig. S3D**). The disruptive mutations G222Y and L226Y exhibited increased auto-activation measured by IκBα degradation (**Fig. 3E-F**) and the upregulation of several

known TNF-induced transcripts (**Fig. 3G**; most pronounced in G222Y) compared with the control P216Y that showed little to no such activity. On balance, mTMD remained the most consistently auto-activated TNFR1 variant, so we focused our further analysis on this construct.

It is known that enforced clustering of receptors or their intracellular domains can activate downstream signaling (26, 38-40). Because receptor over-expression might promote higher-order clustering, it is not entirely surprising that TNFR1 over-expression can initiate signaling in the absence of ligand. To further explore this effect at lower expression levels, we titrated doxycycline to obtain levels of mTMD TNFR1 close to endogenous receptor levels on TNFR1-sufficient MDFs (**fig. S3**). Strikingly, even with levels of mTMD TNFR1 protein that were equivalent to or lower than WT levels, mTMD TNFR1 was still able to rapidly activate NF- κ B and MAPK signaling in the absence of ligand (**Fig. 3H**, compare lane 1 with lanes 6 & 9).

Upon TNF binding, TNFR1 normally recruits a signaling complex, known as complex I, to its cytoplasmic tail, which can contain TRADD, RIPK1 and ubiquitin ligases such as cIAP1 and cIAP2 (18-21, 41-44). Deubiquitinases (DUBs), such as A20/TNFAIP3, are also recruited to complex I to fine-tune and ultimately limit TNF response (45). To see whether the TNFR1 TMD variants recruited complex I in the absence of ligand, we generated C-terminal triple-FLAG-tagged versions. The FLAG-tagged TNFR1 variants were expressed to similar levels and functioned like their untagged counterparts, activating NF- κ B when stimulated with TNF (**fig. S4A**). Yet again, mTMD TNFR1 showed an increased level of ligand-independent activation (**fig. S4B**). Consistent with the previous signaling data, FLAG immunoprecipitations demonstrated increased formation of the TNFR1 signaling complex, with A20 recruitment indicating that mTMD TNFR1 is more auto-activating than wild-type TNFR1 (**Fig. 3I**,

compare lanes 2-4 to 7-9). This auto-activation is even more notable when the much lower amounts of mTMD TNFR1, compared with wild-type receptor, and consequent weaker TNF-induced signal, are considered (**Fig. 3I**, compare lanes 5 and 10; **fig. S4C**). These experiments provide further support for the notion that eliminating specific TMD interactions by replacing the native sequence with mTMD sequence promotes TNFR1 activation in a ligand-independent manner.

The R376K DD self-association mutation prevents mTMD TNFR1 auto-activation

It is believed that ligand-induced receptor oligomerization ultimately activates signaling by promoting aggregation of the intracellular death domain (DD). Despite the conservative nature of the change, mutation of arginine 376 of the DD to lysine (R376K) has been reported to prevent DD self-association in vitro and reduce cell death induced by receptor over-expression in 293T cells (26, 46, 47). To test whether the propensity of mTMD TNFR1 to auto-activate could overcome a DD self-association defect, we generated an R376K TNFR1 mutant in the context of the wild-type and monomeric TMD variants. As before, the mTMD TNFR1 mutant demonstrated an increased propensity to auto-activate when compared with the WT, especially considering its much lower expression level (**Fig. 4A**, compare lanes 7 and 11). However, neither TNF-induced nor mTMD TNFR1 ligand-independent signaling was able to overcome the loss of function caused by the DD mutation, displaying similarly low levels of activation markers by Western blot (**Fig. 4A**, lanes 15 & 16 and 19 & 20). Furthermore, when treated with soluble TNF and compound A (TS), the R376K mutation completely abrogated the ability of both wild-type TNFR1 and TMD mutants to induce TS-dependent apoptosis (**Fig. 4B**).

ECD and TMD interactions have a combined impact on TNFR1 auto-activation.

While the most obvious feature of TNFSF signaling is the fact that trimeric ligand activates signaling via the formation of a trimeric receptor complex, ectodomain (ECD) cleavage of DR5, OX40 and TNFR2 was also reported to be sufficient to initiate signaling (13). This report concluded that TMD oligomerization is inherently activating, and the unliganded ECD normally prevents this. However, when combined with our results, an alternative mechanism is that the ECD acts in concert with the natural TMD structure to prevent DD aggregation, thereby suppressing signaling in the absence of ligand. If this alternative scenario is correct, we reasoned that removal of the ECD would act dominantly to activate both WT and mTMD TNFR1. To test this hypothesis, we generated inducible ECD-truncated (Δ ECD) TNFR1 with the WT or monomeric TMD sequences (**Fig. 5A**) bearing a C-terminal FLAG tag and expressed them in *Tnfr1*^{-/-} MDFs. Loss of ECD reduced TNFR1 expression levels significantly when compared with the full-length versions, but unlike their full-length counterparts, wild-type and mTMD Δ ECD TNFR1 variants activated downstream signaling similarly in the absence of ligand (**Fig. 5B**). The removal of ECD, referred as ectodomain shedding, has been reported to regulate TNF signaling, most probably by producing soluble TNFR1 that captures free TNF ligand thereby reducing activation (48). However, TNFR1 shedding may also lead to rapid degradation of membrane associated TNFR1, causing reduction in cell surface levels of the receptor (49). Therefore, we believe that the dominant effect of physiological TNFR1 shedding is to reduce receptor levels, rather than inducing TNFR1 auto-activation.

The ECD directs, via the PLAD, TNFR1 pre-ligand dimer formation (6, 14, 15, 50). Because the PLAD dimer is inactive, and ligand binding causes only minor changes in the structure of the ECD (7, 14-16), a plausible model predicts that the PLAD dimer structure traps DDs in a specific inhibited state that must be overcome by aggregation into higher-order oligomers where neighboring DDs can interact productively. Similarly, a study on DR5 has shown that

disruption of the pre-ligand ECD association by agonists can induce receptor activation (51).

We reasoned that, if our TMD replacements were acting by weakening the inhibitory effect of this dimer, then blocking its formation would eliminate the auto-activating advantage of mTMD over the WT receptor. To test this hypothesis, we generated a PLAD mutation in the context of WT and mTMD receptors. Lysine 32 is centrally located in the PLAD dimer interface, and mutation to alanine (K32A) prevents pre-ligand assembly of human TNFR1 (6). The K32A mutation in mouse TNFR1 did not reduce TNFR1 expression when compared with its WT counterpart, and neither did it impair TNF-induced activation of NF- κ B and MAPK signaling (**Fig. 6A**). Comparing ligand-free signaling capabilities, the introduction of K32A may have reduced the magnitude of the gap between the native TMD and mTMD receptors (**Fig. 6B**), but it did not change the observation that mTMD exhibited significantly more auto-activation than the native TMD (**Fig. 6B and C**). Similarly, on both WT and K32A backgrounds, mTMD receptors induced equal or greater cell death than those with the native TMD (**Fig. 6D**). This mutation did not therefore provide conclusive evidence for or against a relationship between ECD and TMD interactions in suppression of auto-activation.

There are many naturally occurring mutations in the ECD of human TNFR1 that are associated with TNF receptor-associated periodic syndrome (TRAPS). One such mutation, R92Q, is located at CRD2 of human TNFR1 (24, 25). This mutation is not located within PLAD region (15), does not appear to affect ligand binding or receptor self-association and has similar ectodomain structure compared to wild-type TNFR1 (26, 52). However, it has been reported to exhibit constitutive activation that may be associated with increased flexibility below the PLAD dimer interface (16). To explore its possible cooperative or competitive effects with the TMD, we generated a human ECD, mouse TMD-ICD chimera, and a human ECD-TMD, mouse ICD chimera, because R92 is not conserved in the murine TNFR1 domain. The human

wild-type TMD, similar to mouse wild-type TMD, limited TNFR1 auto-activation compared to our monomeric TMD mutant, and there was no difference between human and mouse wild-type TMD TNFR1 variants (**fig. S6**). Therefore, we generated the R92Q TNFR1 mutant with either the mouse wild-type TMD or mTMD variants (**Fig. 7A**). These human-mouse chimeric TNFR1 (hs-mm TNFR1) variants were expressed in dox-inducible fashion, and cell-surface expression levels were similar (**Fig. 7B**). The chimeric receptors activated signaling normally in response to TNF ligand (**Fig. 7C**) and, consistent with our previous findings with mouse TNFR1, the mTMD chimera mutant showed an increased propensity to constitutively activate NF- κ B and MAPK signaling in the absence of ligand (**Fig. 7C**, compare lanes 7 with 11). The R92Q mutant also enhanced auto-activation when compared with WT (**Fig. 7B**, compare lanes 7 with 15). Furthermore, the combined auto-activation associated with mTMD and R92Q was increased when compared with either alteration alone (**Fig. 7C**, compare lanes 11 or 15 with 19), so much so that activation of this receptor with and without TNF were not distinguishable by any of the biochemical parameters we tested. The R92Q mutation therefore counteracts the inhibitory function of the ECD in a way that is both compatible and additive with disruption of the native TMD interactions.

DISCUSSION

Transmembrane receptors generally require ligand binding to initiate downstream signaling. It is widely accepted that ligand binding changes the receptors from an inactive to an active state either by triggering a conformational change in the intracellular domain (ICD) or by bringing ICDs into close apposition (53). TNFR1 most likely exists as monomers and dimers in cell membranes (6, 11) and is activated by binding of a soluble trimeric TNF ligand (54, 55). This fact suggests that TNFR1 activation requires some form of receptor trimerization, yet TNF binding induces only minor structural changes between the pre-ligand dimer and ligand-bound

trimer ECD structures (16), suggesting that PLAD-driven dimerization and TNF-induced trimerization are not mutually exclusive (56, 57). This has led to a model whereby trimeric ligand binding could bring together a mixture of TNFR1 monomers and pre-ligand dimers to form trimeric and larger complexes whose base units could contain nine, twelve or more receptors, depending on the geometry imposed (9, 11, 16). The stoichiometry in **Figure 8A** illustrates an example where activated trimers and nonamers predominate, based on the cell-surface fluorescence quantitation reported by Karathanasis et al (11).

The TMDs of TNFR1 and at least two other family members (Fas and DR5) are capable of forming trimers, and while these structures are believed to support receptor activation in Fas and DR5, it is not clear what role TMD trimerization plays in TNFR1 activation. To explore the role of TMD oligomerization, we generated TNFR1 variants with natural and artificial TMD sequences possessing different oligomerization potentials. Our most striking finding is that TNFR1 tolerates profound changes in the sequence, structure and oligomeric state of the TMD without notably affecting its ability to activate NF- κ B and MAPK signaling in response to its natural ligand, soluble TNF. However, we also observed that some TMD variants showed increased auto-activation in the absence of ligand, an effect that was most prominent with a TMD designed not to self-associate at all (monomeric, or mTMD). These results suggest that the natural TMD suppresses ligand-free signaling, but they provide no evidence that it supports ligand-induced signaling and they clearly show that TMD trimerisation is not an absolute requirement for it to occur. This is further supported by the activating effects of reported trimer-disrupting mutations introduced into the otherwise native TNFR1 TMD, though the effects of single-amino-acid mutations were generally weaker than those associated with the designed mTMD. This is different from the models derived for Fas (12) and DR5 (13) signaling, wherein formation of a very specific TMD structure is both necessary and sufficient for activation, and

319 this is held in check only by ligand-free ECD interactions. Fas and DR5 have related but distinct
320 functions to TNFR1 and, notably, they can both assemble cell-death-inducing complexes at the
321 plasma membrane while TNFR1 does not (58). Thus, it is possible that different architectures
322 and functions place distinct constraints on the role of the TMD.

323
324 In all of our experiments, we expressed full-length TMD variants of mouse TNFR1 in murine
325 dermal fibroblasts (MDFs) generated from a *Tnfr1*^{-/-} mouse to avoid complications due to co-
326 expression of the wild-type version of the protein. Overall, expression of the different TMD
327 variants was well tolerated and NF-κB, MAPK signaling and cell death responses to
328 exogenously added TNF were very similar. While differing receptor expression levels
329 complicate the comparison of signaling, it is noteworthy that even with lower protein levels,
330 the propensity to auto-activate was most prominent in the mTMD variant. The correlation of
331 decreased protein level and increased signaling supports our conclusion that the differences in
332 auto-activation between WT and mTMD receptors are significant, but it is not clear whether
333 auto-activation affects receptor expression level, for example by promoting intracellular
334 turnover. Remarkably, even expression of the mTMD mutant close to, and indeed lower than,
335 endogenous TNFR1 levels resulted in auto-activation that could be measured both in the
336 formation of the NF-κB/MAPK activating complex and in downstream signaling. All types of
337 TNFR1 auto-activation were completely blocked by the DD self-association mutant R376K,
338 which is consistent with a model in which auto-activation requires DD interactions that are
339 normally prevented by collaboration between ECDs and TMDs (**Fig. 8**).

340
341 Interestingly, the dimeric TMD derived from glycophorin A appeared to be the most effective
342 suppressor of auto-activation in our experiments; on average it was even less activating than

the native TMD in the absence of ligand. This result could point to a crucial role for the PLAD dimer structure in suppression of ligand-free signaling, because reinforcing it with a strong and specific dimeric TMD interaction enhanced this function. This model requires further investigation since there is, to the best of our knowledge, no experimental evidence that the native TNFR1 TMD can drive dimeric as well as trimeric interactions, like DR5 (13), and we do not know whether any of our TMD alterations result in the **complete** reorganization of intact receptors into the specified oligomeric states in the cell membrane. **It is not immediately clear how a pre-ligand dimer, even with mutually reinforcing ECD-TMD interactions creating a rigid structure down to the intracellular membrane surface, could conformationally restrict DD activation with more than 100 amino acids of at least partially flexible sequence intervening. Thus, it is more likely that this TMD-stabilized dimer is somehow the least permissive to spontaneous formation of higher-order receptor oligomers with productive DD interactions. This is consistent with the converse example in our study, where the additive auto-activating effects of the mTMD substitution and the R92Q mutation both “loosen” the structural pathway from ECD through the TMD, which could more easily permit productive higher-order DD interactions (Fig. 8B). It is also possible that R92Q contributes to auto-activation by disrupting a secondary autoinhibitory ECD interaction such as recently reported for DR5 (51).** The weaker auto-activating effects of trimeric and tetrameric designed TMDs could thus reflect the formation of only partially active oligomeric complexes in which some DD interactions are productive and some are not. All of the engineered TMD variants were able to respond to TNF stimulation, presumably by further clustering through multimeric ligand binding.

We also tested the pre-ligand association mutant K32A in the context of the mouse TNFR1, since it is conserved in mouse and human receptors. Although this mutant has been applied to study the binding site of TNFR1 antagonists (56, 59), it is still unclear whether or why the

K32A mutation results in loss of function, as it has not yet been carefully examined in a signaling context. A previous study with this mutation in human TNFR1 indicated that it prevented receptor self-association and TNF ligand binding (6). However, while another confirmed that its ability to dimerize or form higher-order oligomers upon TNF binding was markedly impaired, it also noted that it was barely detectable at the plasma membrane (11). In our hands, the mouse K32A mutant and WT receptors were expressed to approximately equivalent levels in *Tnfr1*^{-/-} cells. However, unlike R92Q, there was no sign that the K32A mutation significantly affected mTMD TNFR1 auto-activation, either in cell signaling or cell death assays, and it activated NF-κB and MAPK signaling pathways when stimulated with TNF like the WT receptor.

In summary, our work demonstrates that no specific TMD structure or oligomeric state is needed to support TNF-induced signaling through TNFR1. Instead, self-associations via the natural TMD and ECD collaborate to prevent productive DD association and subsequent TNFR1 auto-activation. We propose that in the native receptor, these DD interactions are prevented by the natural TMD (**Fig. 8A**) in a way that can be approximated to varying degrees by engineered TMD structures, but not by the monomeric one (**Fig. 8B**). More broadly, this study demonstrates how enforcing a range of defined alternative TMD structures using engineered sequences can reveal mechanistic insights about natural receptor structure-function relationships that are difficult to glean from more traditional loss-of-function mutations. We expect that the de novo designed sequences applied here, and recently elsewhere (27), will prove valuable for mechanistic studies of many other single-spanning receptors.

MATERIAL AND METHODS

Cell lines, reagents and antibodies

Tnfr1^{-/-} MDFs were isolated from *Tnfr1*^{-/-} mouse tails and transformed with SV40 for immortalization. All mouse care was in accordance with relevant ethical regulations and studies were approved by the Walter and Eliza Hall Institute Animal Ethics Committee (Ethics reference number 2018.050). Cell lines expressing TNFR1 variants were generated by infecting *Tnfr1*^{-/-} MDFs with the 2nd generation lentivirus containing dox-inducible TNFR1 coding sequences. Lyophilized human TNF was a gift from D. N. Männel. The smac-mimetic compound A was synthesized by TetraLogic Pharmaceuticals. Phosphor-NFκB p65 (CST, 3033), NFκB p65 (CST, 4764), IκBα (CST, 9242), phosphor-JNK1/2 (CST, 4668), phosphor-MAPK p38 (CST, 4511), RIPK1 (CST, 3493), A20 (CST, 5630), TNFR1 (Abcam, 19139), β-actin (Sigma -Aldrich, A-1978), FLAG (Sigma -Aldrich, F1804) antibodies were purchased.

Recombinant TMD peptide production

For biochemical analysis, the de novo designed tetrameric TMD peptide was expressed to inclusion bodies in *E. coli* BL21(DE3) as a 9His-trpLE fusion with a unique methionine preceding the desired peptide product. Inclusion body extraction, nickel-affinity binding and cyanogen bromide digest were performed as previously described (60) with the addition of 10 mM *S*-methyl methanethiosulfonate (Merck) in the extraction step. The peptide with methanethiol (SMe)-protected C-terminal cysteine incorporated for fluorescent labelling (not used here) was HPLC purified on a ZORBAX SB-300 C3 column (Agilent) and stored as lyophilized product. Samples of defined mass were dissolved directly into NuPAGE lithium dodecyl sulfate sample buffer (Thermo Fisher) for PAGE analysis.

415 **Primers and plasmids**

416 GFP control construct was synthesized by GenScript. Other constructs were generated using
417 standard polymerase chain reaction, restriction enzyme digestion cloning. Clone sequences
418 were verified by Sanger sequencing performed by the Australian Genome Research Facility.
419 Δ ECD TNFR1 was generated by two steps. Firstly, ECD truncated TNFR1 was generated with
420 Δ ECD sense and antisense primers, which does not contain the signal peptide. Then, signal
421 peptide was re-inserted into the construct with SignalP sense and antisense primers. Point
422 mutations were generated as described before (61). Briefly, four primers, template sense (A),
423 template antisense (D), mutation sense (C), mutation antisense (B), were used to generate a
424 single mutation insert. Two DNA fragments were generated by polymerase chain reaction
425 separately using primer AB and primer CD. The second polymerase chain reaction was carried
426 on with product from the first reaction and primer AD. Insert was then subcloned into the
427 pFTRE3G vector using conventional restriction digest cloning. The primers used are listed as
428 follows:

429

430 R376K: A(5'-gaaggatccaccatgggtctccc-3'), D(5'-gagacgtgctacttccattgtcacg-3'), C(5'-
431 cagcccatgaaTTTcatgaactccttc-3'), B(5'-cagcccatgaaTTTcatgaactccttc-3'); K32A: A(5'-
432 gaaggatccaccatgggtctccc-3'), D(5'-cgcggtacctgagtcctgggg-3'), C(5'-
433 cgggatccaccatgggcctctccacc-3'), D(5'-cgcggtacctgagtcctgggg-3'), C(5'-
434 cacagtggaccAggacaccgtgt-3'), B(5'-acacggtgtccTggtccactgtg-3'); Δ ECD sense(5'-
435 cgcggtaccaccatgcccaggactcaggtaccgcg-3'); Δ ECD antisense(5'-
436 ctagctagctcgcgaggcggtcgt-3'); SignalP sense(5'-
437 ggggtacctgagtcctgggggttgacatttgcagggactagcccagtgaccctgatggat-3'); SignalP

438 antisense(5'-cggggtacccccagtgaccctgatggatgtat-3'); hs TNFR1 ECD sense(5'-
439 cgggatccaccatgggcctctccacc-3'); hs TNFR1 ECD antisense(5'-ggggtacctgagtcctcagtcgcct-3')

440

441 **Cell lysis and Western blotting**

442 Cells were lysed in DISC buffer [150 mM sodium chloride, 2 mM EDTA, 1% Triton X-100
443 (v/v), 10% glycerol (v/v), 20 mM Tris-HCl (pH 7.5)], supplemented with phosphatase
444 inhibitors (b-glycerophosphate, sodium molybdate, sodium pyrophosphate and sodium
445 fluoride) and cOmplete protease inhibitors (Roche). Lysates were incubated on ice for 15 min
446 and centrifuged at maximal speed for 10 min. Supernatants were boiled in 5× SDS loading
447 buffer [10% SDS (w/v), 50% glycerol (v/v), 0.1% bromophenol blue (w/v), 200 nM Tris-HCl
448 (pH 6.8)] containing 10% β-mercaptoethanol (v/v), and separated on 4 to 2% gradient SDS–
449 polyacrylamide gels (Bio-Rad), transferred to polyvinylidene fluoride (Millipore) membranes,
450 followed by immunoblotting and detection with indicated antibodies.

451

452 **Immunoprecipitation**

453 anti-FLAG M2 magnetic beads (Sigma-Aldrich) were blocked with 2% BSA in DISC buffer
454 (w/v) at 4°C for 1 hour, and incubated with cell lysates at 4°C overnight. Beads were then
455 washed three times with ice-cold DISC buffer and two times with PBS, followed by elution
456 with 1 mg/ml FLAG peptides at 25°C on shaker. Eluted samples were collected and analyzed
457 by Western blotting.

458

459 **Flow cytometry**

MDFs were seeded at a density of 1×10^4 per well in 96-wells plates 24 hours prior to the treatments. Cells were treated as indicated treatments for the indicated time, and then collected by trypsinization. To measure cell death, dead cells were identified by propidium iodide (PI) staining and cell death was quantified by the percentage of PI positive cells with BD FACSCalibur (BD Biosciences). To analyze TNFR1 surface expression, cells were resuspended in ice-cold FACS buffer [10% FCS (v/v) in DPBS] and transferred into 96-wells U bottom plate, stained with 0.25 μ g hs TNFR1 antibody (R&D Systems, MAB225) or mm TNFR1 antibody (Abcam, 19139) per 1×10^6 cells for 30 min on ice. Cells were then washed twice with FACS buffer and stained with 2 μ g/ml secondary antibody anti-mouse AF488 (Invitrogen, A-11001) for 30 min on ice in dark. Cells were washed twice with FACS buffer and analyzed with BD FACSCalibur (BD Biosciences).

Time-lapse imaging (IncuCyte)

MDFs were seeded at a density of 2,000 per well in 96-wells plates 24 hours prior to the treatments. Dead cells were identified by PI (0.25 μ g/ml) staining. Dyes and compounds were added 10 min before the scanning. Images were taken every 1 hour by time-lapse imaging using the IncuCyte live cell analysis imaging (Essenbioscience) for 24 or 48 hours. Cell death was obtained by normalizing PI positive (dead cells) counts to cell confluency using the built “Basic” analysis software of the IncuCyte. Results were exported as PI counts per area to be graphed using Prism v9.5 (GraphPad).

Supplementary Figs. S1 to S6

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678 the manuscript. MJC contributed to design, and reviewed, edited the manuscript. JS and MEC
679 conceived the project. MEC contributed to design and interpretation, reviewed and edited the
680 manuscript. JS supervised the experiment design and interpretation, reviewed and edited the
681 manuscript. **Competing interests:** The authors declare no competing interests. **Data and**
682 **materials availability:** All data needed to evaluate the conclusions in the paper are present in
683 the paper and/or the Supplementary Materials.

684

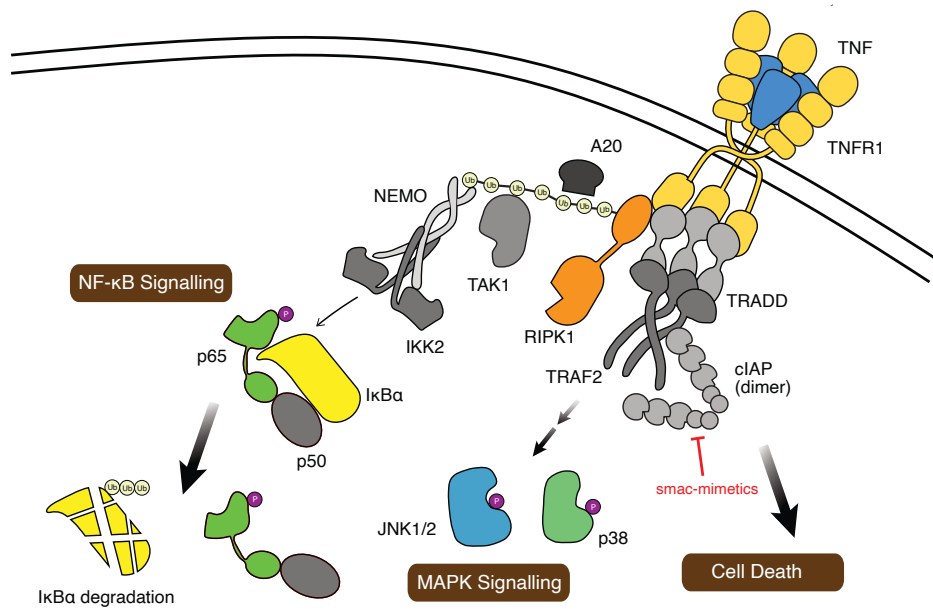


Fig. 1. A minimalistic schematic model of TNFR1 signaling. TNF ligand binding to TNFR1 can induce NF-κB, MAPK signaling and cell death pathway.

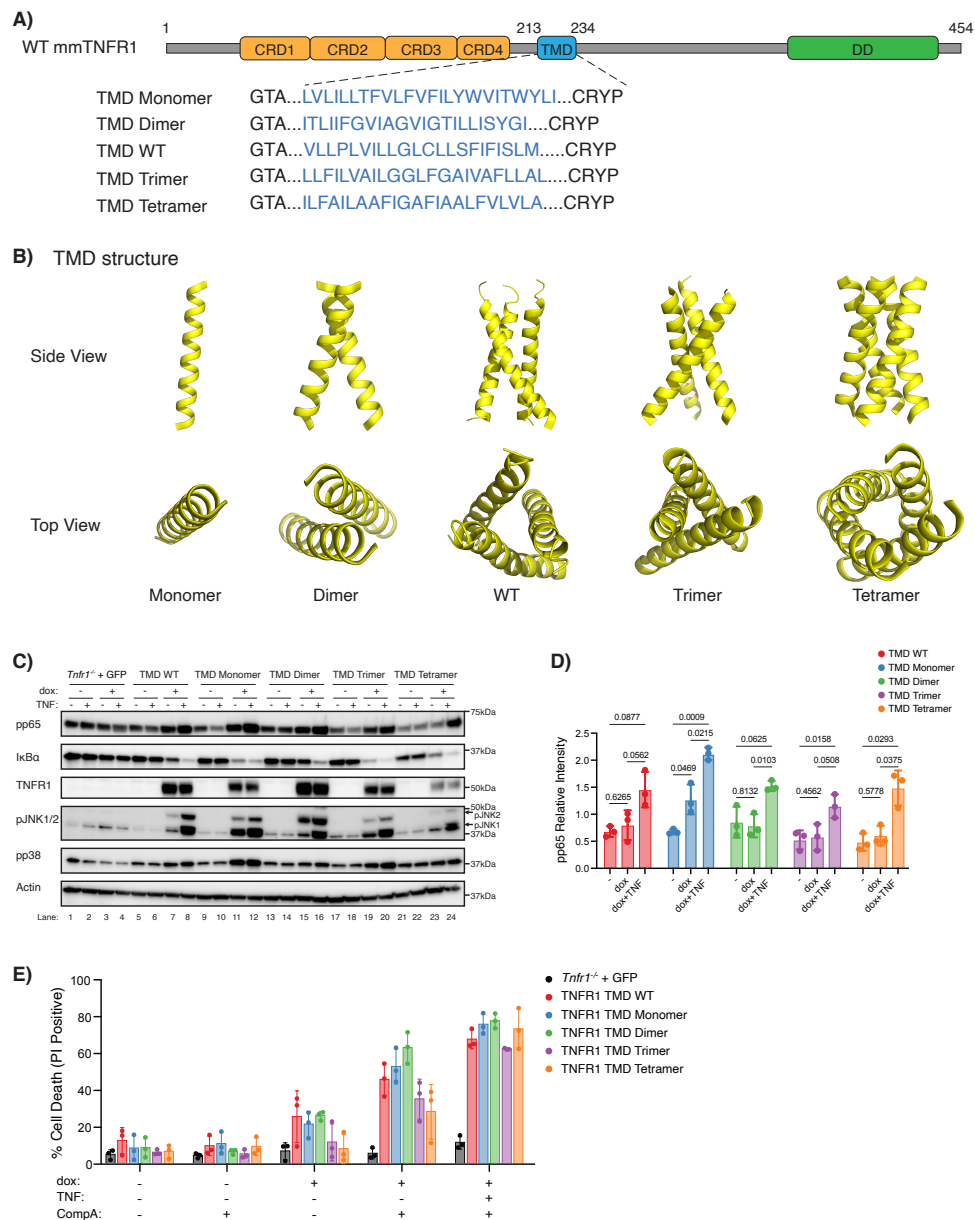


Fig. 2. Altering the oligomeric state of the TMD does not impair ligand-induced TNFR1 signaling. (A) Sequences of monomeric, dimeric, trimeric, tetrameric and wild-type mm TNFR1 TMD. (B) TMD structures in top and side view. Monomer and tetramer are models of de novo designed domains; dimer is adapted from GpA dimer, PDB 5eh4; trimer, PDB 6w9y; WT, PDB 7k7a. (C) *Tnfr1*^{-/-} MDFs expressing dox-inducible TMD variants were pre-treated ± dox (100 ng/ml) for 1 hour, followed by ± TNF (50 ng/ml) for 30 min. NF-κB and MAPK signaling were analyzed by western blot. Blots are representative of 4 independent experiments. (D) Western blot

quantification of pp65 relative intensity from 3 independent experiments. *Tnfr1*^{-/-} MDFs expressing TNFR1 variants were treated ± dox (100 ng/ml) ± TNF (50 ng/ml). ImageLab software version 6.1 was used to quantify western blot band intensities. To enable comparison between different experiments, western blot replicates were normalised by mean of all data in a replicate, and then plotted as bar graph. Paired t tests. (E) *Tnfr1*^{-/-} MDFs expressing dox-inducible TMD variants were pre-treated ± dox (100 ng/ml) for 3h, followed by ± TNF (10 ng/ml) ± CompA (50 nM) for 24 hours. Cell death levels were represented by the percentage of PI-positive cells and measured by flow cytometry. Data are shown as mean ± standard deviation (SD) of 3 independent experiments.

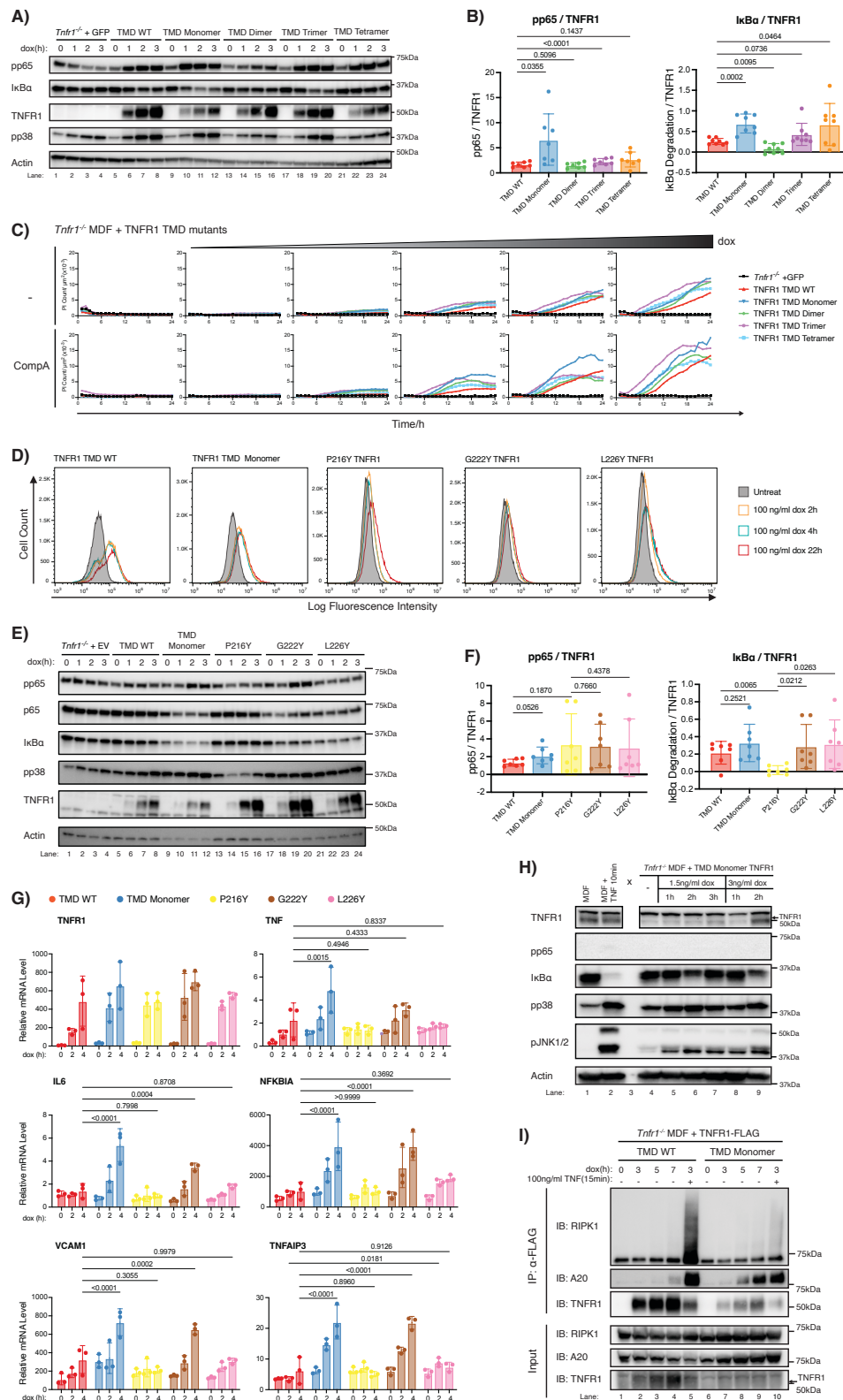


Fig. 3. The monomeric TMD variant (mTMD) promotes TNFR1 auto-activation. TMD variants or TNFR1 single mutations were reconstituted in *Tnfr1*^{-/-} MDFs and induced with dox for all the following experiments. (A) TNF-independent activation of NF-κB

and MAPK signaling assessed by western blot. MDFs were treated with dox (100 ng/ml) for indicated time. **(B)** Western blot quantification of *Tnfr1*^{-/-} MDFs expressing TNFR1 variants treated with dox (100 ng/ml) for 1-3 hours. ImageLab software version 6.1 was used to quantify western blot band intensities. pp65/ TNFR1 was calculated as a ratio of band intensities from treated samples to the relevant untreated (0-hour) time point (*treated/UT*), then divided by the corresponding TNFR1 levels; IκBα/ TNFR1 was determined using the ratio of difference in band intensities between treated and untreated (UT) samples to UT ((UT – treated)/UT), then divided by the corresponding TNFR1 levels. To enable comparison between different experiments, western blot replicates were normalised by mean of all data in one western blot experiment, and then plotted as bar graph. Data of pp65 are from 7 times points in 3 independent experiments; Data of IκBα are from 8 times points in 4 independent experiments. Paired t tests. **(C)** TNF-independent cell death monitored by time-lapse imaging (Incucyte) of PI staining. MDFs were treated with dox (100, 50, 25, 12.5, 6.25, 0 ng/ml) ± CompA (500 nM) for 24 hours. **(D)** Cell surface expression of TNFR1 variants in MDFs. **(E)** Activation of NF-κB and MAPK signaling assessed by western blot. MDFs were treated ± dox (100 ng/ml) for 1 hour, followed by ± TNF (100 ng/ml) for 30 min. **(F)** Western blot quantification as in (B) above. *Tnfr1*^{-/-} MDFs expressing TNFR1 TMD variants or P216Y, G222Y, L226Y mutants were treated ± dox (100 ng/ml) for 1-4 hours. Data are from 7 times points in 4 independent experiments. Paired t tests. **(G)** The relative mRNA levels of TNFR1 and downstream signaling targets in *Tnfr1*^{-/-} MDFs expressing dox-inducible TNFR1 variants. Cells were treated with dox (100 ng/ml) for indicated time. Data are shown as mean ± standard deviation (SD), n = 3, two-way ANOVA, with Dunnett's multiple comparison test. **(H)** mTMD-induced NF-κB and MAPK activation assessed

by western blot. WT MDFs were treated $\pm$ TNF (100 ng/ml) for 10 min, acting as the control. *Tnfr1*^{-/-} MDFs expressing dox-inducible mTMD were treated with low dose of dox (1.5, 3 ng/ml) for indicated time to mimic the endogenous expression level of TNFR1. (I) TNF-induced and TNF-independent complex I immunoprecipitation. *Tnfr1*^{-/-} MDFs expressing dox-inducible C-terminal FLAG-tagged TMD variants were treated $\pm$ dox (100 ng/ml) for indicated time, $\pm$ TNF (100 ng/ml) for 15 min. Complex I was immunoprecipitated by FLAG M2 affinity beads. Western blots and IncuCyte graphs are representative of at least 3 independent experiments.

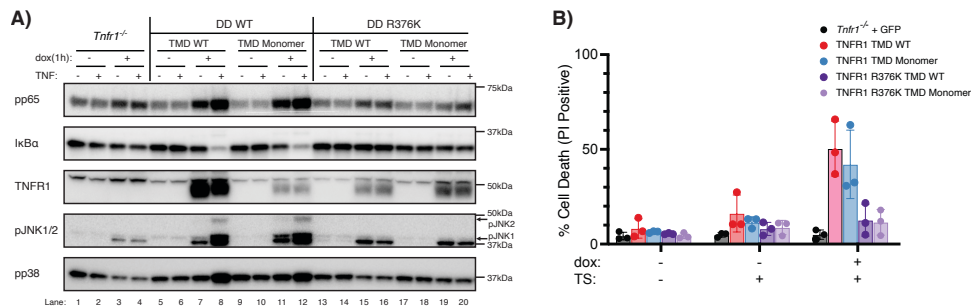


Fig. 4. The R376K death domain self-association mutation prevents TNFR1 auto-

activation. *Tnfr1*^{-/-} MDFs expressing dox-inducible TMD variants with/ without R376K mutation were pre-treated with (A) $\pm$ dox (100 ng/ml) for 1 hour, followed by $\pm$ TNF (100 ng/ml) for 15 min. NF-κB and MAPK signaling were analyzed by western blot. Blots are representative of 3 independent experiments; (B) $\pm$ dox (100 ng/ml) for 3h, followed by $\pm$ TS (10 ng/ml TNF, 50 nM CompA) for 24 hours, cell death levels were represented by the percentage of PI-positive cells and measured by flow cytometry. Data are means $\pm$ SD of 3 independent experiments.

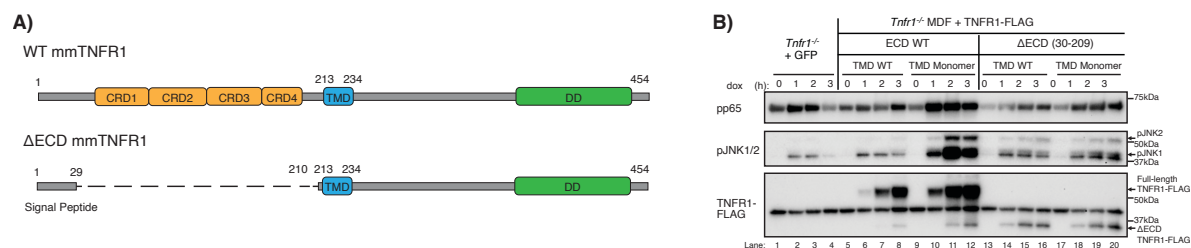


Fig. 5. ECD removal diminishes the difference between WT and mTMD TNFR1

activation. (A) Design of ECD truncated (ΔECD) TNFR1 variants. (B) *Tnfr1*^{-/-} MDFs expressing dox-inducible C-terminal FLAG-tagged WT ECD or ΔECD TNFR1 TMD variants were treated with dox (100 ng/ml) for indicated time. NF-κB and MAPK signaling were analyzed by western blot. Blots are representative of 3 independent experiments.

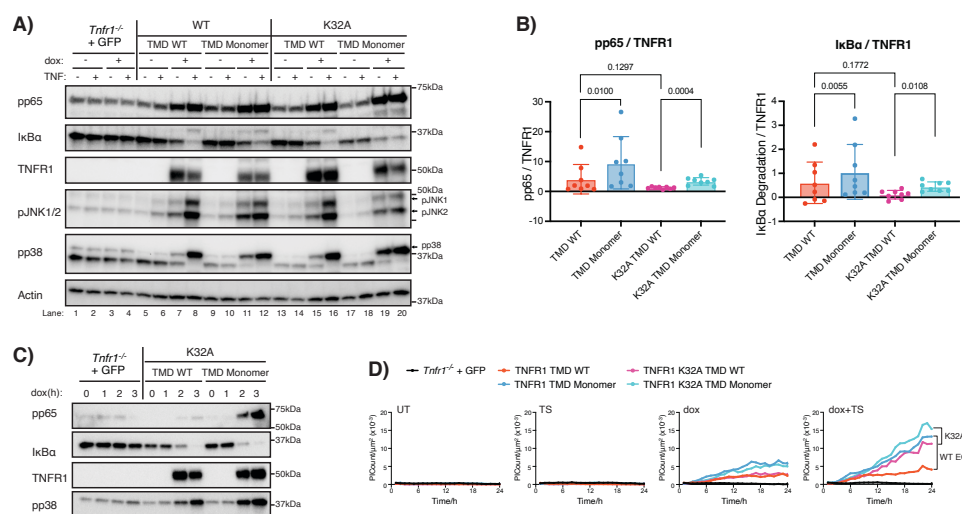


Fig. 6. The K32A mutation does not impair mTMD auto-activation. TMD variants with/

without K32A mutation were reconstituted in *Tnfr1*^{-/-} MDFs, and induced by dox for all the following experiments. (A) Western blots analysis of NF-κB and MAPK signaling. MDFs were pre-treated ± dox (100 ng/ml) for 1h, followed by ± TNF (100 ng/ml) for 15 min. (B) Western blot quantification as described in figure 3B. *Tnfr1*^{-/-}

MDFs expressing WT and monomeric TMD TNFR1 variants with/without K32A mutation were treated with dox (100 ng/ml) for 1-3 hours. Data are from 8 time points in 4 independent experiments. Paired t tests. (C) MDFs were treated with dox (100 ng/ml) for indicated time, and analyzed by western blot. (D) Cell death monitored by time-lapse imaging (Incucyte) of PI staining. MDFs were treated $\pm$ dox (100 ng/ml) $\pm$ TS (10 ng/ml TNF, 50 nM CompA) for 24 hours. Western blots and IncuCyte graphs are representative of at least 3 independent experiments.

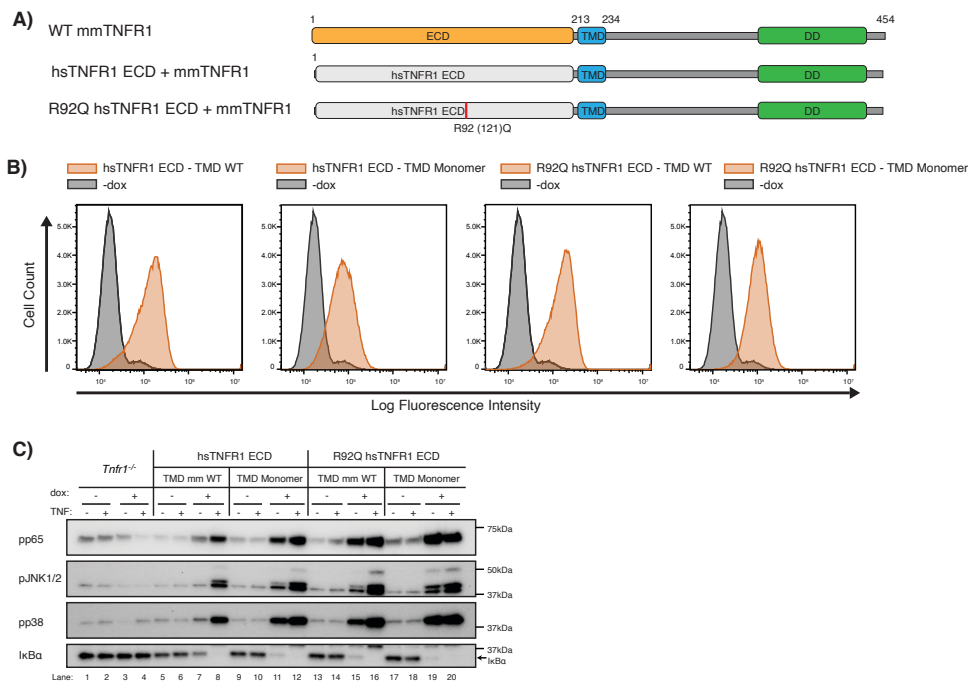


Fig. 7. TRAPS-related mutation R92Q promotes TNFR1 auto-activation. (A) Design of human-mouse chimeric TNFR1 (hs-mm TNFR1) and R92Q mutant. (B) The expression of TNFR1 variants was induced by dox and the surface expression was analyzed using Flow Cytometry. Grey bar represents parental *Tnfr1*^{-/-} MDFs, acting as negative control. (C) *Tnfr1*^{-/-} MDFs expressing dox-inducible hs-mm TNFR1 variants were pre-treated $\pm$ dox (100 ng/ml) for 1h, followed by $\pm$ TNF (100 ng/ml) for 15

min. NF- κ B and MAPK signaling were analyzed by western blot. Blots are representative of 3 independent experiments.

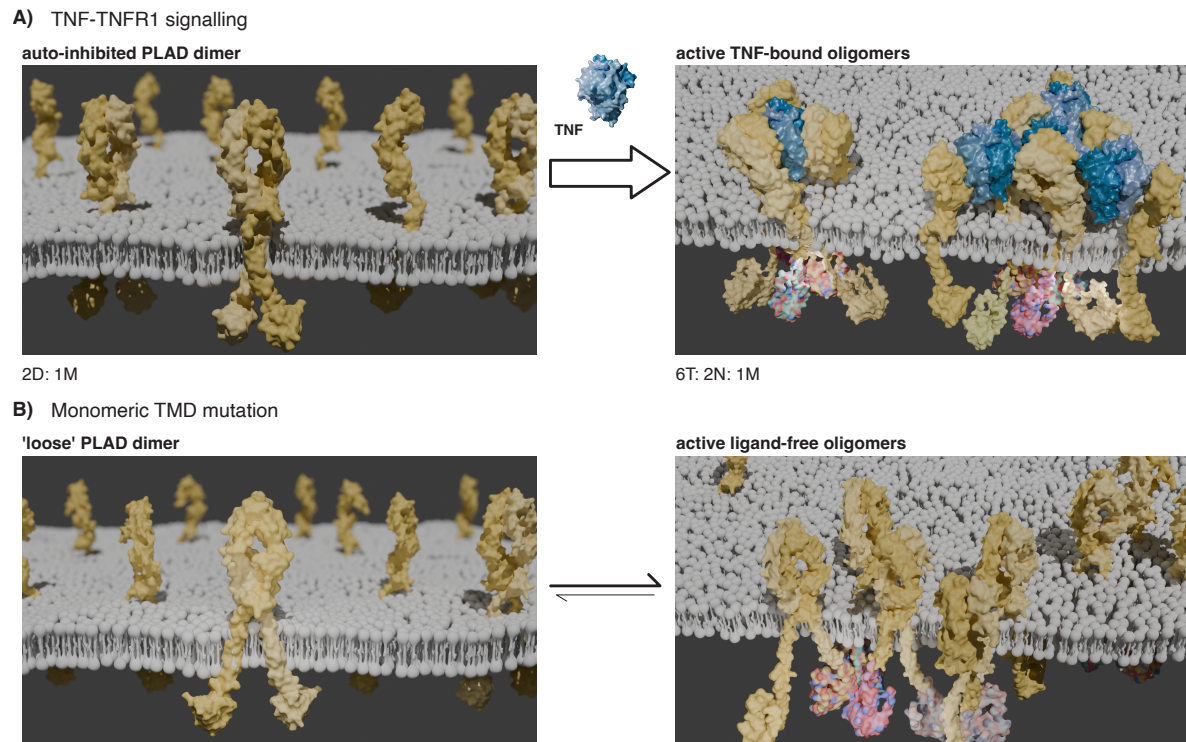
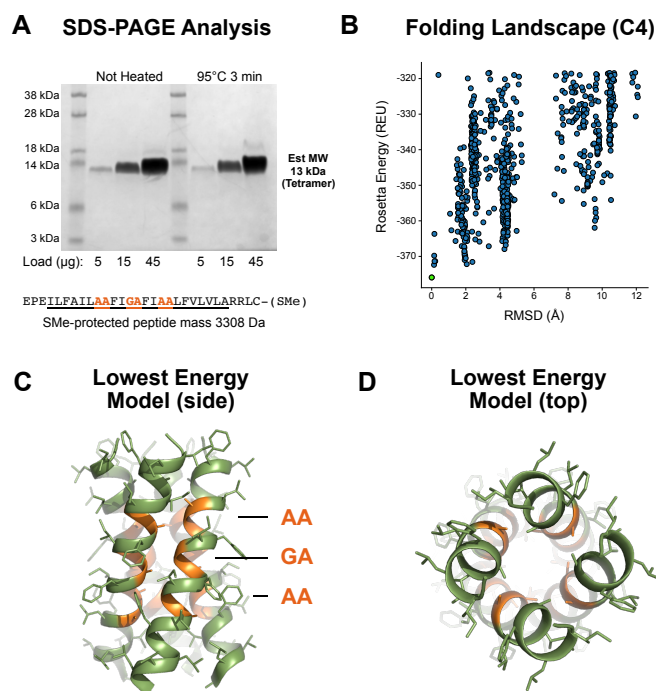


Fig. 8. Models of TNF-induced receptor activation and mTMD TNFR1 auto-activation.

(A) Trimeric TNF ligand induces TNFR1 activation. Pre-ligand TNFR1 (yellow) exists in dimer (D) and monomer (M); trimeric TNF ligand (blue) drives the formation of trimeric (T) and nonameric (N) complexes, where DD aggregation triggers activation. (B) Monomeric TMD TNFR1 has a propensity to auto-activate. The monomeric TMD loosen the structural pathway from PLAD to the intracellular space and permit DD interactions, causing receptor auto-activation.

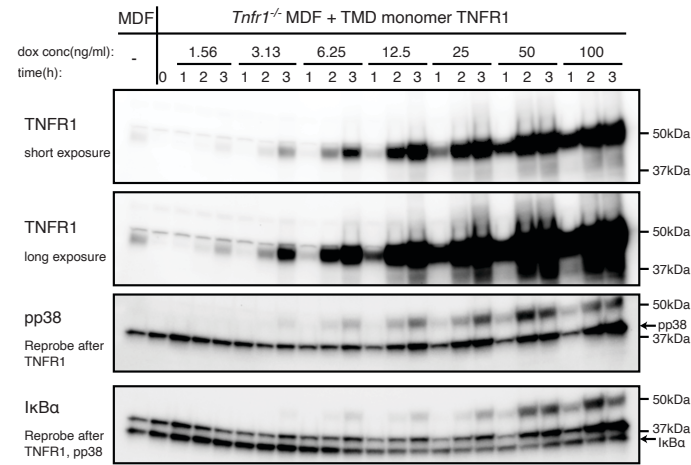


Supplementary Fig. 1. Design and experimental validation of a tetrameric TMD. The tetramer derives from a de novo design campaign for self-associating TMDs described in Elazar et al., eLife 2022 (reference 28) but the sequence has not been previously published. **(A)** Recombinant peptide with the sequence shown below was analyzed by non-reducing SDS-PAGE with and without prior heating of the sample in loading buffer containing 1% dodecyl sulfate. The designed TMD sequence (underlined) is flanked by N-terminal EPE and C-terminal RRLC sequences to facilitate purification and handling. Cysteine is protected by treatment with S-methyl methanethiosulfonate during production to prevent disulfide bond formation. Molecular weight estimated using the MW analysis tool in Image Lab software (Bio-Rad). The actual mass of the monomeric peptide is given below the sequence for reference. **(B)** Ab initio folding landscape using TMHOP (Weinstein PLoS Computational Biology, 2019) of the designed sequence while imposing tetrameric (C4) symmetry. The lowest-energy model (shown in **(C)** and **(D)**) is marked by a green dot, and the RMSD values are computed for all models with respect to this

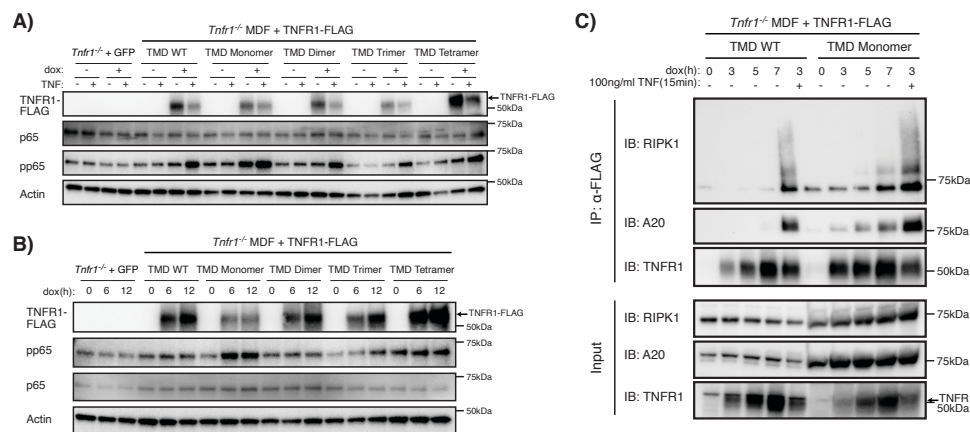
lowest-energy one. Other models exhibit higher energy, indicating that the design is predicted to fold uniquely into the lowest-energy structure.

mm TNFR1 TMD	213	VLL	PLVILL	GLCLL	SFIFISLMC	235
hs TNFR1 TMD	212	VLL	PLVIFF	GLCLL	SLLFIGLMY	236

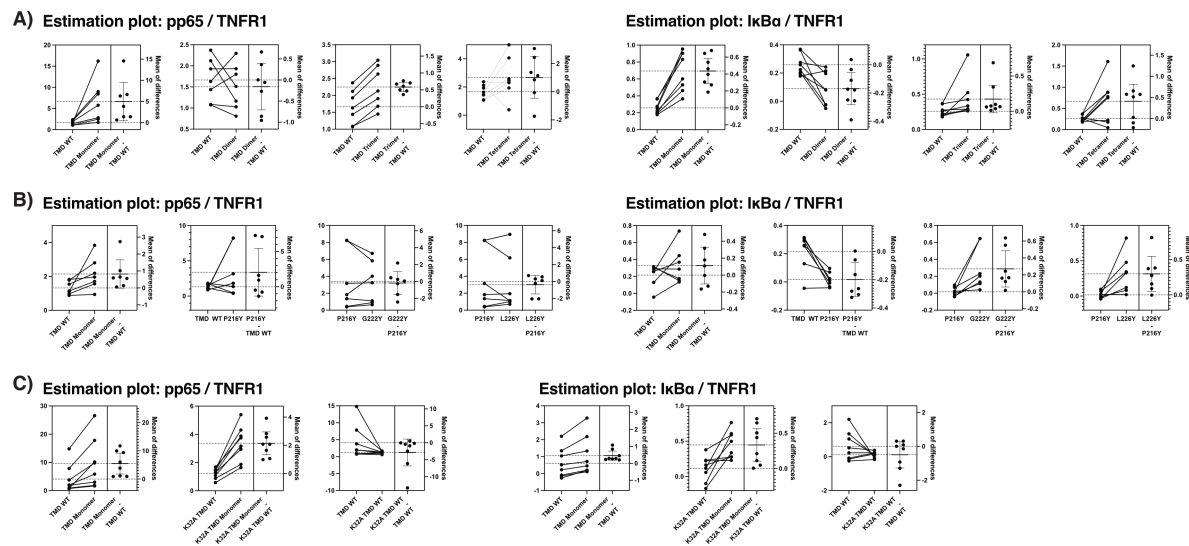
Supplementary Fig. 2. The sequences alignment of wild-type mm TNFR1 and hs TNFR1 TMD.
The P212, G221 and L225 (human numbering) are conserved between human and mouse TNFR1.



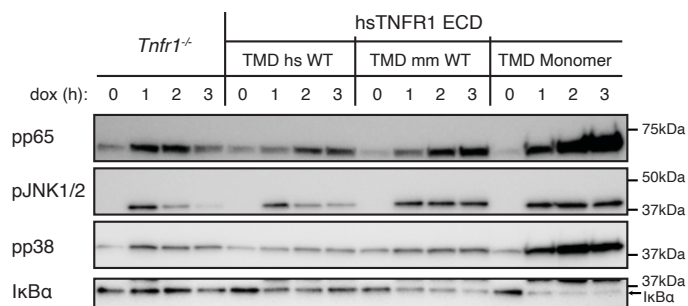
Supplementary Fig. 3. The titration of mTMD expression levels. *Tnfr1*^{-/-} MDFs expressing dox-inducible mTMD were treated with dox of indicated concentration for indicated time. TNFR1 expression level and NF-κB, MAPK signaling were analyzed by western blot.



Supplementary Fig. 4. Activation of FLAG-tagged TNFR1 TMD variants. C-terminal FLAG-tagged version of TMD variants were reconstituted in *Tnfr1*^{-/-} MDFs, and induced by dox for all the following experiments. **(A)** Western blots analysis of NF-κB and MAPK signaling. MDFs were pre-treated ± dox (100 ng/ml) for 3h, followed by ± TNF (50ng/ml) for 30 min. **(B)** TNF-independent activation of NF-κB and MAPK signaling assessed by western blot. MDFs were treated with dox (100ng/ml) for indicated time. Blots are representative of 3 independent experiments. **(C)** Analysis of Complex I formation in Fig. 3D. Samples were normalized according to TNFR1 expression level in cell lysate, so that the expression levels of wild-type and monomeric TNFR1 are comparable.



Supplementary Fig. 5. Paired t test of NF-κB signaling activation. Estimation plot from paired t tests in (A) figure 3B; (B) figure 3F; (C) figure 6B.



Supplementary Fig. 6. Ligand-independent activation of human-mouse chimeric TNFR1. Human ECD-TMD + mouse ICD, human ECD + mouse TMD-ICD chimera receptor, or human ECD monomeric TMD mutant were reconstituted in *Tnfr1*^{-/-} MDFs, and induced by dox (100 ng/ml) for indicated time. Activation of NF-κB and MAPK signaling was assessed by western blot. Blots are representative of 3 independent experiments