TNFR1 membrane reorganization promotes distinct modes of TNFα signaling

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Signaling by the ubiquitously expressed tumor necrosis factor receptor 1 (TNFR1) after ligand binding plays an essential role in determining whether cells exhibit survival or death. TNFR1 forms distinct signaling complexes that initiate gene expression programs downstream of the transcriptional regulators NFκB and AP-1 and promote different functional outcomes, such as inflammation, apoptosis, and necroptosis. Here, we investigated the ways in which TNFR1 was organized at the plasma membrane at the nanoscale level to elicit different signaling outcomes. We confirmed that TNFR1 forms preassembled clusters at the plasma membrane of adherent cells in the absence of ligand. After trimeric TNFα binding, TNFR1 clusters underwent a conformational change, which promoted lateral mobility, their association with the kinase MEKK1, and activation of the JNK/p38/NFκB pathway. These phenotypes required a minimum of two TNFR1-TNFα contact sites; fewer binding sites resulted in activation of NFκB but not JNK and p38. These data suggest that distinct modes of TNFR1 signaling depend on nanoscale changes in receptor organization.

INTRODUCTION
Tumor necrosis factor α (TNFα) is a master proinflammatory cytokine, and dysregulated TNF signaling is implicated in the pathology of a broad range of inflammatory diseases (1). TNFα binds to TNF receptor 1 (TNFR1), which is a member of the TNFR superfamily of which there are 29 known members. Most members of the TNFR superfamily contain cysteine-rich domains (CRDs) in the extracellular domain (2). TNFRs are thought to exist as preassembled oligomers on the cell surface, mediated by the preligand assembly domain (PLAD) that resides within the N-terminal CRD1 that is not directly involved in ligand binding (3). Soluble PLAD can prevent TNFR signaling and inhibit inflammatory arthritis, suggesting that PLAD-mediated receptor assembly is required for TNFR signaling (4). Mutations within CRD1 and CR2 that are thought to render TNFR1 constitutively active are also associated with the inflammatory disease TNFR-associated periodic syndrome (TRAPS) (5, 6). However, crystal structures of TNF-TNFR superfamily complexes, including TNFR1 itself, suggest that individual PLADs are disassociated (7). Further, x-ray structure analysis of the TNF-TNFR2 complex in solution demonstrates that, in the absence of ligand, receptors assemble as parallel or anti-parallel dimers (8, 9). The potential relevance of these solution structures to receptor signaling within intact cells remains unclear as do the potential molecular changes in TNFR1 that occur in response to different context-dependent stimuli.

TNFR1 also contains a death domain (DD) within cytoplasmic tail that recruits adaptor molecules leading to the assembly of signaling complexes (1 and IIa/b/c) that promote distinct functional outcomes (10). After TNF binding, complex I is assembled at the plasma membrane and comprises TNFR1-associated death domain protein (TRADD), receptor-interacting serine/threonine-protein kinase 1, TNFR-associated factor 2 (TRAF2), cellular inhibitor of apoptosis protein 1 (cIAP1) or cIAP2, and linear ubiquitin chain assembly complex (LUBAC). The current evidence suggests that TRAF2 and cIAP1/cIAP2 ubiquitinylate complex I components, and the LUBAC complex adds further linear ubiquitin chains to stabilize and amplify signaling (11, 12). Recruitment and activation of the transforming growth factor–β–activated kinase 1 (TAK1) complex and the inhibitor of κB kinase (IKK) complex then activate unique downstream effectors. TAK1 is involved in activating mitogen-activated protein kinase (MAPK) signaling cascades that lead to activation of JUN N-terminal kinase (JNK), p38, and AP1 transcription factor, whereas IKKβ activates the canonical nuclear factor κB (NFκB) pathway (11, 13). Induction of signaling complex I leads to the expression of AP1 and/or NFκB target genes that are important in inflammation, cell proliferation, and survival. However, the way in which TNFR1 complexes are fine-tuned to initiate differential signaling upon assembly of complex I remains unknown.

TNFR1 is proposed to be a preformed dimer at minimum but assumed to form higher-ordered clusters on ligand binding to initiate signaling (3). Furthermore, cholesterol-rich lipid raft domains and palmitoylation modifications may be required for TNFR1 signaling after ligand binding by promoting coalescence of preassembled TNFR1 clusters to form functional signaling platforms (14, 15). However, lipid rafts are not required for TNFR1-induced NFκB signaling responses to ligand (16, 17). Given the essential role of TNFR1 in mediating cell behavior under homeostatic and inflammatory conditions, understanding the way in which TNFR1 assembles and signals at the plasma membrane is of central importance to defining the role of this receptor in disease settings. Here, we used a combination of biochemical and advanced microscopy approaches in adherent cells to define the role of TNF-TNFR1 interactions in promoting receptor clustering and specific signaling events. Our findings revealed that TNFR1 formed preassembled clusters at the plasma membrane of adherent cells in the absence of ligand, and this was independent
of cholesterol and extracellular TNFα interactions but partly dependent on the presence of the cytoplasmic domain. Trimeric TNFα binding induced conformational change within the ectodomain of TNFR1 leading to lateral TNFR1 cluster mobility, TNFR1-MEK1 association, and initiation of JNK/p38/NFκB activation. These phenotypes all required a minimum of two TNFR1-TNFα contact sites, as fewer binding sites initiated NxFκB activation but not JNK/p38. These data demonstrate that distinct modes of TNFR1 signaling depend on nanoscale changes in receptor organization and conformation.

RESULTS

TNFR1 forms ligand-independent higher-ordered clusters at the plasma membrane

To determine the organization of TNFR1 at the plasma membrane, we first analyzed the localization of TNFR1 in adherent cells using both widefield and total internal reflection fluorescence (TIRF) microscopy. Images demonstrated that, in starved HeLa cells, TNFR1 was clustered within the cytoplasm around the perinuclear region with small, irregularly positioned clusters of TNFR1 at the plasma membrane (fig. S1A). This preclustered localization of TNFR1 at the plasma membrane was also observed in a range of other adherent cell types (fig. S1B). To allow us to study the behavior of these TNFR1 clusters in live cells, we generated TNFR1 knockdown HeLa cells (fig. S1C) and reexpressed full-length, wild-type (WT) TNFR1–green fluorescent protein (GFP) in these cells. Expressed TNFR1-GFP bound to TNFα (fig. S1D) showed very similar colocalization at the plasma membrane as endogenous TNFR1 (Fig. 1A and fig. S1E) and restored TNF-dependent signaling to NxFκB in TNFR1-depleted cells (figs. S1, F and G). We next tested whether the size or number of TNFR1 clusters at the plasma membrane changed in response to TNFα treatment. Analysis of TIRF images of TNFR1-GFP or those counter-stained for TNFR1 demonstrated no change in the distribution of cluster sizes in cells after treatment (Fig. 1A), and a very similar result was obtained from images of endogenous TNFR1 in parental HeLa cells (fig. S1H). Together, these data indicated that TNFR1 was constitutively clustered at the plasma membrane in adherent cells, and TNFα binding did not alter the size or distribution of these clusters, as measured by diffraction-limited imaging.

To determine whether the extracellular regions of TNFR1 contributed to the preformed clusters at the plasma membrane, we expressed three different mutant forms of TNFR1-GFP in TNFR1 knockdown HeLa cells: R92Q (within CRD2) or C52F (within CRD1), previously characterized in patients with TRAPS (18), and a quadruple-point mutation within CRD1 predicted to destabilize putative preformed associations through CRD1 (Q17A/K19A/H34A/D49A). When we compared the distribution of each receptor mutant to WT TNFR1 by TIRF microscopy, we found no substantial change in plasma membrane clustering with any of the mutants compared to WT TNFR1 (Fig. 1B). We also expressed TNFR1-GFP lacking the cytoplasmic domain (ΔCD) to determine whether preformed clusters were instead due to formation of associated complexes at the cytoplasmic face of the receptor. In the absence of the CD, TNFR1 diffusely localized at the plasma membrane and was still able to assemble into discrete clusters (Fig. 1B). Moreover, coexpression of WT and ΔCD or AAAA mutants of TNFR1 revealed no colocalization between the WT and mutant receptors at the membrane (Fig. 1C), suggesting that specific conformations of TNFR1 may preferentially co-associate into membrane clusters. These data suggest that formation of TNFR1 clusters in the absence of ligand may not depend on previously reported regions within the extracellular domains (19) but that the presence of the cytoplasmic tail can potentially stabilize these higher-ordered complexes at the plasma membrane.

Our data showed no change in TNFR1 cluster size or number in cells after ligand binding using diffraction-limited microscopy methods. To determine whether interactions between individual receptors were altered at the nanoscale, we analyzed fluorescence resonance energy transfer (FRET) between coexpressed GFP- and RFP-labeled WT TNFR1 homodimers using fluorescence lifetime imaging microscopy (FLIM). In agreement with our TIRF images, FRET efficiency analysis demonstrated a high degree of preformed clustering in starved cells but a significant increase in direct TNFR1 homo-interactions after TNFα binding (Fig. 1D) that corresponded with a shift to lower lifetimes across the receptor population (fig. S2A). In cells expressing ΔCD TNFR1, we observed similar interactions between receptors, which were not increased by ligand binding (Fig. 1D). There was no detectable FRET between WT TNFR1 and ΔCD TNFR1 with or without TNFα (fig. S2C), which confirmed that TNFR1 WT and mutant forms preferentially homo-oligomerize.

Nanoscale organization of TNFR1 preassembled clusters changes after ligand binding

Our FRET data demonstrated that TNFR1 homo-oligomerization increases after TNFα treatment but without changes in the overall cluster size or number by diffraction-limited microscopy. To determine whether TNFR1 may undergo exchange between discrete clusters during activation to drive this increased homo-oligomerization, we performed fluorescence recovery after photobleaching (FRAP) analysis of cells coexpressing TNFR1-GFP and TNFR1–monomeric red fluorescent protein (mRFP). TNFR1-GFP was bleached and recovery analyzed after TNFα stimulation while simultaneously following the unbleached TNFR1-RFP to accurately track the cluster. Recovery curves demonstrated no new TNFR1-GFP were recruited to clusters after activation (Fig. 2A). These data suggest that there is no detectable exchange of receptors between clusters after ligand binding. To further define the potential changes to TNFR1 organization within clusters, we used stochastic optical reconstruction microscopy (STORM) to quantify positioning of individual TNFR1 molecules at the plasma membrane before and after ligand binding. From the detected individual localizations, we determined that TNFα stimulation of cells did not change the number of localizations per area (Fig. 2, B and C) or the percentage of localizations identified that were positioned within categorized high-density (Fig. 2D) or low-density (Fig. 2E) clusters. However, a significant increase in the proportion of TNFR1 clusters containing high-density, closely packed molecules was observed in TNFα-activated compared to starved cells (Fig. 2F). These data are in agreement with our FLIM data showing increased receptor proximity under these conditions (Fig. 1D) and suggest that TNFR1 forms preassembled clusters, which increased in local density at the nanoscale after TNFα binding.

TNFR1 clusters undergo dynamic repositioning on the plasma membrane after ligand binding

Our data indicated that ligand binding altered the organization of TNFR1 clusters, similar to other receptor families after activation (20, 21). We next analyzed whether this was also associated with movement of the clusters using time-lapse imaging by TIRF microscopy. We found that TNFR1 clusters moved slowly within the plasma membrane in unstimulated cells, but TNFα treatment significantly
increased cluster movement speed (Fig. 3A). Further analysis of particle tracks demonstrated no correlation between cluster size and movement speed (Fig. 3B). Because TNFR1 is internalized in certain cell types to promote apoptosis (22–24), we next addressed the extent of endocytosis in HeLa cells after TNF binding. We found that ligand binding did not promote TNFR1 internalization for up to 2 hours after treatment using biotin pulse chase experiments (Fig. 3C). To further confirm that endocytosis was not required for TNFR1 activation, we treated cells with the dynamin2 inhibitor Dynasore and analyzed activation of NFκB and JNK. Inhibiting dynamin function had no impact on activation of either pathway (Fig. 3D) or localization of TNFR1 (fig. S3C). These data demonstrated that endocytosis did not play a role in TNFR1 activation under these conditions tested in adherent cells and is therefore unlikely to account for the TNFR1 cluster movement we observed. We also addressed the possibility that TNFR1 ectodomain cleavage may contribute to cluster movement, as TNFR1 has previously been shown to undergo cleavage within the ectodomain by the tumor necrosis factor–α converting enzyme (TACE) (25, 26). However, Western blot analysis for TNFR1 cytoplasmic domain after ligand stimulation did not reveal lower molecular weight bands, and no change in TNFR1 banding pattern was seen after pretreatment with the TACE inhibitor TAPI-0 (fig. S3A). These data indicated that TNFR1 does not undergo cleavage events that might explain the cluster movement we observed under these conditions within the time frames analyzed.

The organization of the plasma membrane into microdomains may alter TNFR1 partitioning and subsequently promote signal transduction from the plasma membrane in some cell types (16, 27). To determine whether cholesterol-rich microdomains partition TNFR1 in adherent cells, we first stained cells with cholera toxin to determine whether TNFR1 localized to GM1-containing lipid rafts. We found no overlap between TNFR1-GFP and Ctx-Alexa555 in starved or TNFα-treated cells, and TNFR1 clusters were still present in cells treated with the cholesterol-depleting agent methyl-β-cycloextran (MBCD) (Fig. 3E).

![Fig. 1. TNFR1 assembles into preformed clusters at the plasma membrane.](http://stke.sciencemag.org/)
TNFR1 clusters also did not colocalize with clathrin (fig. S3B), and altered localization was not altered by treatment with cytochalasin D, which disrupts the actin cytoskeleton, or Nocodazole, which disrupts microtubule polymerization (fig. S3C). TNFα treatment did not stimulate cleaved caspase (fig. S3D), which indicates that TNFα-induced cluster movement does not trigger apoptosis in these cells, and is in agreement with the notion that internalized receptors predominantly initiate cell death. These data combined demonstrated that preformed TNF1 clusters did not reside in cholesterol-rich microdomains in these adherent cells and did not require the cytoskeleton for preassembly.

To determine whether the cytoplasmic regions of TNF1 clusters contributed to the cluster movement, we performed TIRF time-lapse analysis of cells expressing ΔCD-TNF1. We observed that, in comparison to WT TNF1, plasma membrane localization of the mutant receptor lacking the cytoplasmic region increased. Despite this, ΔCD TNF1 assembled clusters that exhibited higher basal movement and did not alter speed in response to ligand binding compared to WT TNF1 (fig. S3E). These data suggest that association between TNF1 cytoplasmic domains may act to stabilize TNF1 clusters. However, time-lapse analysis of TNF1 cluster movement at the membrane demonstrated that cells pretreated with MβCD failed to increase speed in response to TNFα binding (Fig. 3F).

These data suggest that cholesterol-rich microdomains may not tether TNF1 clusters but rather potentially are required to promote interactions with other molecules within the membrane and induce subsequent signaling responses. To test this hypothesis, we analyzed activation of p65 (a subunit of the NFκB complex), p38, and JNK as the key mediators of downstream signaling from active TNF1 by Western blot. Treatment of cells with MβCD had no effect on activation of p65 in response to TNFα, but relative activation of both p38 and JNK significantly reduced compared to dimethyl sulfoxide (DMSO)–treated controls, as previously reported in nonadherent macrophages (17) (Fig. 3G). Our combined data suggest that TNF1 in adherent cells forms preassembled clusters, which require movement within the membrane to trigger specific downstream signals.

**TNF1 cluster movement correlates with ligand-receptor interaction number and signaling**

To further define whether movement of TNF1 clusters could be controlled by ligand binding, we quantified cluster movement in response to treatment of cells with homotrimeric TNFα or where S162T/Y163A mutations were introduced into one, two, or all three TNFα copies (mB, mBC and mABC, respectively). Analytical size exclusion chromatography (AnSEC) analysis of TNF1-TNFα binding confirmed the expected stoichiometry of receptor-ligand interactions for the single (mB) or double (mBC) mutated TNFα compared to WT (fig. S4, A to C). Analysis of time-lapse movies demonstrated that treatment of cells with WT and mB TNFα triggered TNF1 cluster movement

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**Fig. 2. TNF1 increases nanoscale clustering in response to ligand binding.** (A) Confocal microscopy of TNF1-GFP- and TNF1-RFP-expressing HeLa cells before and after photobleaching. Images (left and middle) are representative of three independent experiments. Quantified fluorescence recovery of TNF1-GFP in clusters over time is means ± SEM of 15 cells from all experiments. (B to F) STORM analysis of GFP clusters in TNF1-GFP–expressing HeLa cells treated with TNFα, as indicated. Images (B) are representative of three independent experiments. Quantification of total GFP area (C), the percentage of GFP localized within high-density (D) or low-density (E) clusters, and the percentage of GFP-containing high-density clusters of total clusters (F) with median values ± upper and lower percentiles of at least 16 cells are pooled from all experiments. Scale bar, (A) 10 or (B) 1 μm. *P < 0.01 by Students two-tailed t-test.
on the membrane, whereas mBC and mABC TNFα did not (Fig. 4A). These data indicated that two TNFR1 receptors need to be engaged with ligand under these conditions to induce movement of clusters on the membrane. To determine whether altered cluster speed correlated with altered signaling (as seen in MjCD-treated cells), we analyzed activation of p65, p38, and JNK in lysates from cells treated with WT or mutant homotrimERIC TNFα. Whereas mB and mBC TNFα induced equally strong p65 phosphorylation when compared to WT ligand, mBC TNFα stimulated reduced amounts of pp38 and pJNK (Fig. 4B). Furthermore, mABC TNFα did not induce a signaling response in any of the pathways analyzed (Fig. 4B). To define the phenotypic effects of treatment with these forms of TNFα, we analyzed cytokine secretion using an enzyme-linked immunosorbent assay–based array. We found that WT TNFα promoted the secretion of macrophage inflammatory protein–1α, CD40L, CXCL11, and granulocyte-macrophage colony-stimulating factor by HeLa cells, but only some of these changes were stimulated by B or BC mutants (fig. S5, A and B). However, the proliferation rates in cells treated with the different TNF forms were unchanged over 48 hours (fig. S5C), which suggested that altered cytokine release was not acting in an autocrine manner. As a strategy to explore this question further, we analyzed activation of p65, p38, and JNK in lysates from cells treated with TNFα analyzed activation of p65, p38, and JNK in lysates from cells treated with TNFα and/or MjCD as indicated. Blots (left) are representative of five independent experiments. Quantified band intensity values (right) are means ± SEM from all experiments. Scale bars, 10 µm. *P < 0.01, **P < 0.005, and ***P < 0.001 by two-way ANOVA.

To determine whether TNFR1 receptor movement and binding to at least two adjacent TNFR1 molecules. (A) TIRFM analysis of TNFR1-GFP in HeLa cells treated with WT or mutant TNFα, as indicated. Representative color-coded tracks from 10-min movies are shown. Images are representative of three independent experiments. White lines denote plasma membrane boundary, and color scale indicates track position over time (black, 0 min; yellow, 10 min). Quantified TNFR1-GFP cluster movement speed data with medians ± quartile values of 30 cells are from all experiments. (B) Western blots for pp65, pp38, and pJNK in HeLa cells treated with WT or mutant TNFα, as indicated. Blots (top) are representative of five independent experiments. Quantified band intensity values (bottom) are medians ± quartile values from all experiments. (C) FRET analysis in cells coexpressing WT TNFR1-GFP and WT TNFR1-RFP untreated or treated with mutant TNFα. Data are medians ± SEM of 30 cells from three independent experiments. (D) STORM analysis of TNFR1 clusters from HeLa cells treated with WT or mutant TNFα. Images are representative of three independent experiments. (E) TIRFM analysis of TNFR1 (green) and MEKK1 (magenta) in TNFR1-GFP HeLa cells treated with WT or mBC TNFα. Images (upper) are representative of three independent experiments. White lines denote plasma membrane boundary. Colocalization coefficient data are means ± SEM from all experiments. Scale bars, 10 μm. *P < 0.01, **P < 0.005, ***P < 0.001, and ****P < 0.0001 by two-way ANOVA.

not sufficient to induce TNFR1 interactions that promote cluster movement. To confirm this, we used STORM to quantify the degree of TNFR1 nanoscale clustering under the same conditions. We observed a significant increase in localizations of TNFR1 within high-density clusters in WT TNFα–treated cells but not those stimulated with mBC TNFα (Fig. 4D). Together, these data demonstrate that the number of interactions between TNFR1 and TNF can alter local receptor interaction, which are necessary to activate distinct signaling pathways.

Once activated, TNFR1 can recruit a range of different molecular adaptors and kinases that contribute to differential functional endpoints. To further determine whether TNFR1 clustering and movement correlated with recruitment of specific proteins to the cytoplasmic domains of TNFR1, we performed coimmunoprecipitation (co-IP) of TNFR1 with known binding partners of complex I and its associated downstream kinases. We found that the abundance of TRAF2, silencer of death domains (SODD), RIP, and FADD in complex with TNFR1 was unchanged in cells after TNFα treatment, both for TNFR1–GFP and endogenous TNFR1 (fig. S5, A and B), which suggests that these may exist as preformed assemblies in adherent cells. However, analysis of TNFR1
colocalization with the MAPK family member MEKK1 demonstrated that TNFα promoted a significant increase in association (Fig. 4E). Moreover, this enhanced colocalization between TNFR1 and MEKK1 was not seen in cells pretreated with MJ5CD or those treated with mBC TNFα (Fig. 4E). This TNF-dependent association was not seen with MLK3 (also MAPKKK11), which colocalized with TNFR1 in all conditions, including cholesterol-depleted cells (Fig. S6C). These data suggest that coupling between TNFR1 and MEKK1 correlates with TNFα-TNFRI cluster movement in adherent cells and the differential activation of p38 and JNK (28).

**TNFR1 undergoes an ectodomain conformational change in response to ligand binding**

Our data showed that TNFR1 clusters require interactions with at least two binding sites on the TNFα trimer to induce movement, which correlated with MEKK1 binding and activation of p38 and JNK. However, the molecular changes within TNFR1 clusters regulating this movement and capture of MEKK1 remain unclear. We hypothesized that, when two or more TNFα molecules bind TNFR1, this may induce a conformational change within the ectodomain that promotes TNFR1 movement and a full complement of signaling activation. To test this in intact cells, we performed FRET/FLIM analysis to quantify the proximity of the extreme N-terminal region of TNFR1 to the plasma membrane. Given that we observed no difference in receptor movement or clustering in response to ligand-binding in WT TNFR1 and ΔCD TNFR1-expressing cells, we first analyzed the potential for TNFα to induce changes to WT or ΔCD TNFR1. FLIM analysis demonstrated that a population of TNFR1 was in proximity to the plasma membrane in unstimulated cells, with no differences between WT and ΔCD TNFR1 (Fig. 5A). However, TNFα stimulation resulted in a significant increase in WT TNFR1 proximity to the plasma membrane, which was not seen in ΔCD TNFR1 (Fig. 5A). These data suggest that ligand-binding may promote shortening of the ectodomain of WT TNFR1. To determine whether this altered conformation was dependent on the number of TNFR1-TNFα contact sites, we performed the same experiments in cells stimulated with mBC TNFα. We found no change in FRET in these cells, which demonstrated that trimeric interactions between TNFR1 and ligand are required for close membrane association (Fig. 5B). Together, these data suggest that ligand binding may induce a conformational change within the extracellular domains of TNFR1 clusters that promotes closer association with the plasma membrane.

**DISCUSSION**

TNFR1 is a key receptor in cellular decision-making between growth and inflammation or death. A large body of work over the past three decades has documented the signaling modes that can be triggered downstream of ligand binding to TNFR1. However, the very early events that occur within TNFR1 oligomeric structures that dictate these signaling decisions have remained unclear. Moreover, TNFR1 networks are presumed to be the signaling-competent architecture; the current model for receptor activation suggests that ligand binding drives receptor trimerization, triggering reorganization of the cytosolic domains and higher-ordered network formation (29). Through combining high-resolution imaging and analysis of signaling endpoints, we showed that TNFR1 assembles into discrete preformed clusters at the plasma membrane of adherent cells (summarized in Fig. 5C). These clusters are unchanged in PLAD or putative dimerization mutants within the TNFR1 CRD1/2 domains but are less abundant after removal of the cytoplasmic domain. Although the PLAD domain is important in constitutive dimerization or oligomerization or TNFR1 (3, 4, 18), our data would suggest that larger-scale membrane assemblies of TNFR1 are less reliant on PLAD extracellular domain interactions. Instead, the association with cytoplasmic proteins through the presence of the DD may play a more important role in this process. Using both FRET/FLIM and STORM analysis, we showed that this proximity was further increased upon binding of TNFα to preformed clusters, without altering the overall size of the clusters. STORM can provide molecular mapping of molecules at the ~20-nm scale, and FRET enables detection of interactions below 10 nm (30, 31). Whereas many molecular details of intact TNFR1 are well described (32), the potential molecular distances within preformed or ligand-induced TNFR1 groups have not been previously analyzed in intact adherent cells. Our combined high-resolution imaging approaches have therefore provided insight into receptor proximity and potential conformational changes that correlate with signaling outputs.

Our data demonstrate that changes at both the cytoplasmic and extracellular domains of TNFR1 are required for the tight receptor associations that correlate downstream kinase activation and inflammatory cytokine production. The increased proximity of the TNFR1 N-terminal region to the plasma membrane that we observe here only occurs upon engagement with trimeric TNF that binds two or three receptors. This is coupled with an increased proximity of the cytoplasmic tails and increased clustering within larger-scale preformed membrane assemblies of TNFR1. Although the structural details that underpin these combined conformational changes remain to be defined, our data would suggest that TNFα engages at least two receptors, which promotes increased TNFR1 packing and flattening of the larger-scale TNFR1 cluster with respect to the plasma membrane. Although CRD1 is not required for ligand binding, this domain instead may act to stabilize a conformation of CRD2 that is permissive for ligand-induced activation of TNFR1 (7, 33). In our membrane-proximal FRET experiments, the increased interaction would be predicted to be at the N-terminal portion of CRD1, suggesting that CRD1 also undergoes a conformational change within the receptor molecule that may promote ligand-receptor stabilization and thus full activation.

Although biochemical cross-linking approaches suggest that TNFR1 is a preformed trimer (3), studies using mutated TNFR1 ligands do not support similar conclusions (34). Our FRET and STORM data would also suggest that TNFR1 is at minimum a preformed dimer, as ligand interactions between adjacent receptors within these preformed clusters appear to be sufficient to induce larger-scale assemblies that correlate with receptor activation, as has been previously suggested by molecular modeling experiments (32). The preassembled receptor clusters through cytoplasmic domain interactions would provide spatial proximity sufficient to engage two ligands simultaneously, thus initiating rapid receptor movement and capture of specific signaling molecules. Our data further suggest that a single TNF-TNFR1 ligand interaction can be sufficient to induce activation of NFκB pathways but not JNK and p38. Our data revealed that TNFR1 cluster movement strongly correlated with increased colocalization between TNFR1 and MEKK1, which can initiate p38 and JNK signaling after stimulation by ligand (28, 35). Whereas we did not explore activation of every potential TNFR1-dependent
pathway in this study, our data imply that the changes we observed are not required to trigger IKK signal initiation and further suggest that differential ligand binding or receptor proximity may provide means to fine-tune the signaling response in different physiological contexts. The data we present in this study focus on the effects of soluble TNFα on adherent cells. However, TNFα can also exist as a less well-studied trimeric 26-kDa membrane tethered form (mTNFα), which can elicit both shared and distinct bioactivities when compared to the 17-kDa cleaved, soluble form (36). In contrast to soluble TNFα, mTNFα acts in a juxtacrine fashion through cell-cell contact, which may present the ligand to TNFR in a different conformation, and maintains contact for a longer duration to initiate different signaling outcomes. Applying the approaches we present in the current study to analyze TNFR-mTNFα interactions in the future may provide new insight into the shared and distinct modes of action of these cytokine family members.

Our data demonstrate that, in adherent cells, preformed TNFR1 clusters do not depend on actin or microtubule cytoskeletons and do not localize to lipid rafts or caveolae. Whereas B and T cell receptors require an intact underlying actin cytoskeleton to initiate and propagate signaling in response to ligand binding (37, 38), our study suggests that the cytoskeleton may not be the key factor that enables TNFR1 activation under the conditions tested. Moreover, although biochemical fractionation experiments suggest that TNFR1 resides in lipid rafts microdomains (27), these domains are not required for signaling to occur (16, 17). It remains unclear how the preformed TNFR1 clusters are established at the plasma membrane and whether additional factors or microdomains within the membrane contribute to cluster stability. However, our FRAP data showed no substantial movement of receptors between clusters or internalization of TNFR1 with or without the presence of ligand. This suggests that associations between cytoplasmic domains of adjacent TNFR1 molecules transported to the membrane, and subsequent binding to adaptor proteins, may provide sufficient means to stabilize preformed receptor complexes. Our data also indicated that TNFR1 clusters moved within the membrane after TNF binding in adherent cells. This mobility within the membrane did require the presence of cholesterol and a minimum of two TNF molecules interacting with adjacent TNFR1 molecules. This suggests that TNFR1 needs to undergo additional conformational changes to increase receptor proximity, potentially at minimum as a dimer within a larger network, and this promotes cluster movement.

Fig. 5. TNFR1 undergoes a conformational change in the ectodomain in response to TNFα binding. (A) FRET lifetime analysis of TNFR1 membrane proximity in HeLa cells expressing WT or ΔCD HA-TNFR1 that were treated with TNFα as indicated and stained for HA (green) and DHPE (blue). Images (left) are representative of three independent experiments. Quantified FRET efficiency data (right) are means ± SEM of 45 cells from all experiments. (B) FRET lifetime analysis of TNFR1 membrane proximity in HeLa cells expressing HA-TNFR1 that were treated with WT or mutant TNFα as indicated and stained for HA (green) and DHPE (blue). Images (left) are representative of three independent experiments. Quantified FRET efficiency data (right) are means ± SEM of 30 cells pooled from all experiments. (C) Model of potential TNFR1 ectodomain conformational changes under different ligand binding states. Scale bars, 10 μm. *P < 0.01, **P > 0.005, and ***P < 0.001 by two-tailed t test (A) or two-way ANOVA (B).
defining the mechanisms that control this movement and how this relates to TNFR1 conformational changes. Our study provides insight into the assembly requirements on both sides of the plasma membrane that precedes TNFR1 receptor movement and subsequent initiation of JNK/p38 signaling cascades. In addition to demonstrating that differential nanoscale TNFR1 clustering correlates with signaling outcomes, this approach to defining how TNFR1 behaves under different physiological conditions may help refine anti-TNF and anti-TNFR1 therapeutics for inflammatory disease.

MATERIALS AND METHODS

Antibodies and reagents

Anti-TNFR1 antibody (Mab225) was from R&D systems. Anti–hemagglutinin (HA), anti-phosp65 (Ser529), anti–p65, anti–pJNK, anti–JNK, anti–p38, anti–MLK3, anti–MEKK1, anti–RIP, anti–TRADD, anti–TRAF2, and anti–clathrin antibodies were from Cell Signaling Technology. Anti–Myc antibody (clone 9E10) was from Sigma–Aldrich. Anti–GFP antibody was from Roche. Anti–GFP chromobody conjugated to Alexa647 was from Chromotek. Anti–HSC70 and anti–TNFR1 (H5) were from Santa Cruz Biotechnology. Anti–SOD2 was from Novus Biologicals. Anti–mouse horseradish peroxidase (HRP) and anti–rabbit–HRP were from DAKO, and anti–mouse–568, anti–rabbit–568, and phalloidin–647 were all obtained from Invitrogen.

Plasmids

TNFR1–YFP, TNFR1ΔCD–YFP, TNFR1–R92Q–YFP, and TNFR1–C52F–YFP were a gift from R. Siegel [National Institute of Arthritis and Musculoskeletal and Skin Diseases/National Institutes of Health (NIH), Bethesda, USA]. GFP-tagged versions of these constructs were generated by subcloning TNFR1 sequences into eGFP–N1 (Clontech). EmGFP– and TagRFP-tagged versions were generated by subcloning EmGFP or TagRFP into NheI and XbaI of GFP–N1 plasmid. EmGFP DNA was generated by polymerase chain reaction (PCR) and then inserted into GFP–N1. TNFR1 Q17/K19/H34/D49 AAAA–EmGFP and TagRFP were generated by site–directed mutagenesis using the following PCR primers. Q17A, 5′GAGATAGGGATGTGTGTCCCGCAGGAAATATATATCCAC3′ (forward) and 5′GGTGGATATATTTTCTGCAGGACACACTATCTC3′ (reverse); K19A, 5′GTGTTGTCCTCAAGGAGCATATATCACCACCTC3′ (forward) and 5′GGAGGTGGATATATGCTCTTTGGAACAC3′ (reverse); H34A, 5′GATTGTTGACTACAGAATGTCGCCCAAGGACCTATGGAACAC3′ (reverse); D49A, 5′CGCCCGGCGAGCTCAGGACCGAGGAGG3′ (forward) and 5′ACGTACGGCATCCGGACCGGCGCCGG3′ (reverse).

Fused heterotrimERIC human TNFα was designed such that three copies of the human gene (UniProt P01375) were cloned in tandem separated by Ser–Gly–Ser linkers. The first copy of TNFα encodes residues 77 to 233, whereas the second and third copy encodes residues 86 to 233. The construct was optimized for Escherichia coli expression (Gene Composer), synthesized (ATUM), and cloned into the arabinose-inducible expression vector pEMB54 containing a 6His–tag at the N terminus. Receptor–binding mutants were generated by introducing mutations S162T/Y163A (P01375 numbering) into the second copy of TNF (mB), second and third copies of TNF (mBC), or all three copies of TNF (mABC) using the QuikChange II Site–Directed Mutagenesis Kit (Agilent, Santa Clara, CA).

TNFα protein expression and purification

E. coli TOP10 cells (Thermo Fisher Scientific) transformed with each of the expression constructs were cultured in terrific broth (TB) (ampicillin, +100 μg/ml) to an OD600 (optical density at 600 nm) = 0.6, expression was induced by adding arabinose (0.1%), and cultures were incubated for a further 16 hours at 20°C. Cells were harvested by centrifugation and stored at –80°C. Cells were resuspended (1 g in 4 ml) in 25 mM tris–HCL (pH 8.0), 200 mM NaCl, 0.02% CHAPS, 50 mM L–arginine, 125 U of Benzonaze (Novagen), 100 μg of lysozyme, and one Complete EDTA–free protease inhibitor tablet (Roche) and lysed by sonication. Insoluble material was removed by centrifugation, and His–tagged protein was captured from the soluble fraction by immobilized metal affinity chromatography (IMAC) (HiTrap Chelating HP, GE Healthcare) eluted with a 500 mM imidazole step or gradient. The 6His–Smt tag was removed with ubiquitin–like–specific protease 1 while dialyzing against 2 liters of 25 mM tris (pH 8.0) and 200 mM NaCl overnight at 4°C in 10-kDa molecular weight cut–off (MWCO) snakeskin dialysis tubing. Cleaved protein was further purified by a second IMAC step, followed by size exclusion chromatography (HiPrep 16/60 Sephacryl S–100 HR, GE Healthcare) in 10 mM Hepes (pH 7.5) and 150 mM NaCl.

Analytical size exclusion chromatography

WT and mutant forms (mB and mBC) of fused trimer hTNFα were incubated for 1 hour at 22°C with hTNFR1 at 1.2–2.2–3.2– and 3.5-fold molar excess over fused trimer (final concentrations: hTNFR1, 90, 165, 240, and 375 μM; hTNFα, 75 μM). Samples were subjected to analytical size exclusion using high–performance liquid chromatography. Injection volumes of 50 μl were separated on a TSK G3000SW l × 1D. Column (30 cm by 7.5 mm, 10 μm in particle size) pre–equilibrated in 10 mM Hepes (pH 7.5) and 150 mM NaCl.

Cell culture

HeLa cells were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS) supplemented with penicillin and streptomycin. TNFR1 knockdown HeLa (HeLa–SH3) cells were generated using pSP–u6(n) plasmids containing short hairpin RNA targeting the 5′ untranslated region of TNFR1 mRNA, which were obtained from Creative Biogene (NY, USA), and were maintained in HeLa growth medium supplemented with puromycin (1 μg/ml). Normal human keratinocytes were cultured in keratinocyte serum–free medium (Invitrogen) supplemented with penicillin and streptomycin. HT1080 cells were a gift from V. Sanz–Moreno (King’s College London, UK) and were maintained in DMEM containing 10% FCS supplemented with penicillin and streptomycin. The fibroblast cell lines NIH–3T3 were obtained from ATCC and were maintained in DMEM containing 10% FCS supplemented with penicillin and streptomycin.

Western blotting and IP

HeLa cells (1 × 10⁶) per condition were cultured in normal growth medium and lysed in 100 μl of sample buffer containing β–mercaptoethanol at room temperature. Lysates were immediately subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and blotted using nitrocellulose membrane. Blots were blocked and probed using 3% bovine serum albumin (BSA)/tris–buffered saline–0.1% Tween or 3% milk/phosphate–buffered saline (PBS)–0.1% Tween. For IP experiments, HeLa were transfected with TRAF2–myc or HeLa–SH3 transfected with TNFR1–GFP and cultured for a further
counts per well were calculated for each time point. EVOS2-FL fluorescent microscope (Thermo Fisher Scientific, UK). fixed at either 24 or 48 hours after treatment, followed by DAPI cubated in serum-free media with no treatment or containing WT

Cytokine array

HeLa cells were grown to 60% confluency in six-well plates, media were changed to Opti-MEM (Gibco, UK) with or without the addition of noted single chain TNFα (scTNFα) (10 ng/ml) and incubated for 8 hours. Media was then removed and assayed using the Human Cytokine Array Proteome Profiler Array (R&D Systems, Abingdon, UK) according to manufacturer’s instructions. Samples were then analyzed for mean spot pixel intensity by densitometry, and resulting data were presented as fold change compared to untreated control samples.

Proliferation assays

HeLa cells were plated at 2.5 × 10⁴ cells per well in serum-free media and left overnight to adhere. Five wells per condition were then incubated in serum-free media with no treatment or containing WT or mutant TNF (10 ng/ml). Cells were placed into the incubator and fixed at either 24 or 48 hours after treatment, followed by DAPI (4’,6-diamidino-2-phenylindole) staining and imaging on an EVOS2-FL fluorescent microscope (Thermo Fisher Scientific, UK). Tile scans of every well were reconstructed in Fiji, and nuclear counts per well were calculated for each time point.

Sample preparation for confocal and TIRF microscopy

HeLa cells cultured on fibronectin-coated 13-mm coverslips were washed with PBS, fixed with 4% paraformaldehyde (PFA) in PBS for 10 min. For detection of intracellular proteins, cells were also permeabilized with 0.2% Triton X-100 for 10 min before antibody incubation. Cells were incubated with primary antibodies for 2 hours, appropriate secondary antibodies were conjugated to Alexa Fluor 568 or Alexa Fluor 647, and phalloidin was conjugated to Alexa Fluor 568 or 647 for 1 hour. Cells were mounted onto slides using Immunofluore (ICN).

For TIRF analysis, HeLa cells cultured in fibronectin coated eight-well glass bottom chambers (Ibidi) were washed with PBS and fixed with 4% PFA in PBS for 10 min. For detection of intracellular proteins, cells were also permeabilized with 0.2% Triton X-100 for 10 min before antibody incubation. Cells were incubated with primary antibodies for 2 hours, and appropriate secondary antibodies were conjugated to Alexa Fluor 568 or Alexa Fluor 647. For TNF546 labeling, TNFR1-GFP–transfected SH3 were incubated on ice for 20 min with TNF-546 (10 ng/ml) in PBS before fixation with 4% PFA. TIRF images were acquired in PBS using a Nikon A1R microscope with TIRF capability using a CFI Apo TIRF with 1.48 numerical aperture (NA) 60× oil objective (Nikon) and a cooled charge-coupled device (CCD) camera (Hamamatsu).

Analysis of fixed TIRF images

TIRF images were analyzed for colocalization of TNFR1-GFP puncta and indicated proteins using JACoP plugin in Fiji (imagej.nih.gov/ij/plugins/track/jacop.html). Briefly, corresponding green and red images were thresholded using the JACoP plugin to select membrane-associated puncta. Colocalization was then analyzed using Manders and plotted as mean values across at least 20 images per condition.

Time-lapse TIRF microscopy and image analysis

For live tracking of TNFR1-EmGFP clusters, HeLa transfected with TNFR1-EmGFP were cultured in eight-well glass bottom chambers (Ibidi) in imaging medium supplemented with 25 mM Hepes. Images were acquired using a Nikon A1R microscope with TIRF capability using a CFI Apo TIRF with 60× oil objective 1.48 NA (Nikon). Temperature in the chamber was controlled to 37°C using an environmental chamber and control unit (Okolab). Cells were then imaged every 30 s using 488-nm laser excitation with perfect focus system (PFS) activated. All images were saved as .nd2 files and analyzed using the TrackMate plugin (ImageJ). A Python script was written in-house to analyze the relationship between cluster area and speed of movement in the live cell TIRF microscopy data. To segment the images, we followed a similar approach to that described in (39) and applied wavelet filtering, followed by watershed segmentation to identify the clusters. After segmentation, the centroid position and area of each cluster can be determined. The cluster centroids were tracked using a Python implementation of the particle tracking algorithm first developed in (40) (https://zenodo.org/record/34028, 10.5281/zenodo.34028). Tracks with a minimum length of 10 frames were retained, and their mean square displacement was calculated to allow determination of the cluster diffusion coefficient.

FRET/FLIM

For FRET experiments, fibronectin-coated eight-well glass bottom chambers of HeLa-SH3 transfected with TNFR1-EmGFP and TNFR1-TagRFP plasmids were treated with TNFα (10 ng/ml) for 5 min before fixation with 4% PFA and treatment with 0.1% Triton X-100 and then sodium borohydride (1 mg/ml). FLIM was used to measure FRET between EmGFP and TagRFP, which allows the determination of spatial protein interactions. Time domain FLIM was performed with a multifocal multichannel microscope system, as described previously (41). Briefly, light generated from a Chameleon Ultra II Ti:Sapphire laser source (Coherent Inc.) is coupled with a spatial light modulator to generate a uniform 8 × 8 array of beamlets. This beamlet array is then relayed through a set of galvanometer scanners (providing x-y raster scanning capability) onto the back pupil plane of a 40× Plan Fluor oil objective 1.3 NA (Nikon) where it is projected onto the sample. The two-photon generated fluorescence is collected and descanned where it is directed with a dichroic mirror and focused onto the Megaframe single-photon avalanche diode (SPAD) array using a 10× Plan Fluor air objective 0.3 NA (Nikon). For each individual image acquisition, the system processed 64 × 64 data points for 8 × 8 detectors producing 512 × 512 pixel images. Lifetime data were acquired by operating the Megaframe camera in time-correlated single photon counting (TCSPC) mode. In TCSPC mode, on-pixel time-to-digital converters (TDCs) generate raw time-correlated data, which are stored and then post-processed offline to generate an image. Once processed, these data are saved and then subsequently analyzed using TR2 lifetime analysis software.

For FRET experiments measuring TNFR1 ECD conformational change, fibronectin-coated glass coverslips of HeLa-Sh3 transfected with HA-TNFR1 were treated with TNFα (10 ng/ml) for 5 min before fixation with 4% PFA. Cells were blocked with 5% BSA–PBS and then immunostained with anti-HA antibody (Cell Signaling Technology) diluted 1:800 in 5% BSA–PBS, followed by a Fab fragment directly conjugated to Alexa Fluor 488 secondary antibody diluted 1:1000 in 1% BSA–PBS without cell permeabilization. Coverslips were
Fluorescence lifetime imaging capability was provided by time-correlated single-photon counting electronics (SPC-830) on DCC-100 control (both from Becker & Hickl). A 40× Plan Fluor oil objective was used throughout (1.3 NA; DIC H, WD 0.2; Nikon), and data were collected at 515 ± 20 nm through a bandpass filter. Acquisition times of the order of 250 s at a low 900-nm excitation laser power (MaiTai, DeepSee; Spectra-Physics) were used to achieve sufficient photon statistics for fitting while avoiding either pulse pile up or photobleaching. Corresponding widefield fluorescence images were acquired for the acceptor [N-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoetheanolamine (DHPE), TexasRed] channel (DS-Qi1Mc camera; Nikon). Lifetime raw data were analyzed with TRI2 software (Paul Barber), and histogram data were plotted as mean FRET efficiency from at least 30 cells per sample over three experiments. Alexa Fluor 488 has been previously shown to fit to a biexponential lifetime with the longer lifetime (43). Thus, a biexponential fluorescence model was used to fit the data using in-house exponential fitting software (TRI2) using a Levenberg–Marquardt algorithm, with the larger value interpreted as that of the Alexa Fluor 488 dye lifetime. Average lifetimes were calculated by the mean of all pixels measured within each image per cell and pooled from multiple experiments for statistical analysis. All graphs are plotted as mean FRET efficiency from >30 cells in total pooled from at least three independent experiments. Lifetime images of exemplary cells are presented using a pseudocolor scale, whereby blue depicts normal Alexa Fluor 488 lifetime (i.e., no FRET) and red depicts reduced Alexa Fluor 488 lifetime (areas of high FRET). Analysis of variance was used to test statistical significance between different populations of data.

**STORM analysis**

For dSTORM experiments, fibronectin-coated glass bottom dishes of HeLa TNFR1 knockdown cells transfected with TNFR1-EmGFP and TNFR1-TagRFP plasmids were treated with TNFα (10 ng/ml) for 5 min before fixation with 4% PFA and treatment with 0.1% Triton X-100. Cells were then blocked with 5% BSA-PBS containing 0.2% Tween for 60 min at room temperature before incubation with anti-GFP chromobody coupled to Alexa Fluor 647 diluted 1:800 in 5% BSA-PBS with Tween 20 (PBST) for a further 60 min. Cells were then washed three times with PBST and three times with PBS to remove residual antibody. Samples were then placed in STORM imaging buffer (glucose oxidase, catalase, 1M MEA) before imaging. STORM measurements were performed on a customized STORM microscope, built around a DMi8 microscope body and “SuMo” passively stabilized stage (Leica Microsystems GmbH). In this system, the 1.43 NA 160× oil objective (Leica Microsystems GmbH) is mounted to the underside of the stage through a piezo drive (PI). Diode lasers of 638 nm (Vortran), 561 nm (Oxxius), 473 nm (Dragon Laser), and 405 nm (Vortran) as appropriate were depolarized through optic fibers, combined, apertured, and expanded to pass through the objective and provide TIRF illumination. The TIRF beam that reflected back through the objective was picked off with a half mirror and imaged on a 128-photodiode microarray (RS). The signal was digitized and centroided by a microcontroller (Arduino). Focus drift caused displacement of the reflected beam on the array. This drift was monitored and corrected for using the piezo drive. Fluorescence was split according to wavelength by an image splitter (Photometrics Dual-View) and imaged side by side on a fast EMCCD camera (Photometrics Evolve). For GFP, the filter window used was 500 to 530 nm (the “green” channel), and for Alexa Fluor 647, it was 660 to 695 nm (the “red” channel). This was achieved with a multilane major dichroic and emission filter set, a beam splitter, and interference filters (Semrock). Low-intensity 473-nm light was used to excite the GFP, whereas high-intensity (ca. 7 kW/cm²) excited the Alexa Fluor 647. Acquisitions were consisted of 10,000 10-ms frames. “Snapshots” of the cluster positions in the green channel were taken with a single 1-s exposure frame just before acquisition (or alternatively made from integrating the first 100 frames of the acquisition). Acquired data were saved as TIFF files and processed in ImageJ using the ThunderSTORM plugin. The resulting localization tables were then imported into cluster density software.

**Cluster analysis**

Acquired data were saved as TIFF files and processed in ImageJ using the ThunderSTORM plugin (44). The localization tables were postprocessed to retain only high-quality localizations and to remove background. Localizations were filtered to retain those with a determined precision less than 30 nm, and repeated localizations were merged using a maximum radius of 20 nm X and 5 Y maximum off frames. The filtered localizations were grouped into clusters based on the local density. This was done by first forming Voronoi diagrams as in (45) and (46) and subsequently analyzing the local localization density using software written in-house using the Python programming language. The Voronoi diagram or tessellation was formed by partitioning the field of view into polygons, where there is exactly one polygon for each localization, such that any point within a polygon is closer to the localization than to any other point. The area of each polygon is then a direct indicator of the local density of each localization, polygons have small areas in dense regions. Clusters were defined by measuring the area of every polygon and retaining neighboring polygons where the area is smaller than a preset threshold and where the number of localizations within the region exceeds a minimum occupancy. The area threshold was determined as in (46) by comparing the experimental data with a simulated spatially random distribution of points with the same average density as the experimental data. Monte Carlo simulations were used to produce an average probability distribution of polygon sizes for the spatially random simulated data, and this is compared with the distribution of polygon sizes in the experimental data. The intersection of the two distributions was used as a first-pass automatic threshold to form a set of clusters from the experimental data. The retained localizations were then clustered a second time with a more stringent density threshold to cluster any small, dense subregions of the first-pass clusters.

**Statistical analysis**

Data were analyzed using Prism software (GraphPad).

**SUPPLEMENTARY MATERIALS**

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Fig. S1. TNFR1 clusters occur in multiple adherent cell types.
Fig. S2. TNFR1 molecules increase homotypic associations in response to ligand binding.
Fig. S3. TNFR1 clusters do not require membrane microdomains or cytoskeleton for assembly.
Fig. S4. AnSEC analysis of titration of TNFR1 with scTNFα.
Fig. S5. Differential cytokine release in response to mutant scTNFα binding.
Fig. S6. TNFR1 clusters do not change association with complex I proteins or MALT3 in response to TNF.


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Organizing TNFR1 signaling

The inflammatory cytokine tumor necrosis factor–α (TNFα) stimulates both cell death and survival by activating its ubiquitously expressed membrane receptor, TNFR1. Morton et al. used various microscopy techniques to investigate TNFR1 membrane organization. In resting cells, TNFR1 was found within clusters that required its cytoplasmic tail. After TNFα binding, TNFR1 clusters were denser and moved within the membrane more rapidly, which correlated with the activation of specific downstream pathways and physical association with the kinase MEKK1. Experiments with engineered ligands that could only bind a specific number of TNFR1 molecules suggested that engagement of two receptors was sufficient for signaling but that trimeric interactions were necessary for extracellular conformational changes in TNFR1. These findings suggest how membrane organization alters TNFR1 signaling, insights that may direct the development of TNF-targeted therapeutics with increased potency.