Structural principles of tumor necrosis factor superfamily signaling

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The tumor necrosis factor (TNF) ligand and receptor superfamilies play an important role in cell proliferation, survival, and death. Stimulating or inhibiting TNF superfamily signaling pathways is expected to have therapeutic benefit for patients with various diseases, including cancer, autoimmunity, and infectious diseases. We review our current understanding of the structure and geometry of TNF superfamily ligands, receptors, and their interactions. A trimeric ligand and three receptors, each binding at the interface of two ligand monomers, form the basic unit of signaling. Clustering of multiple receptor subunits is necessary for efficient signaling. Current reports suggest that the receptors are prearranged on the cell surface in a “nonsignaling,” resting state in a large hexagonal structure of antiparallel dimers. Receptor activation requires ligand binding, and cross-linking antibodies can stabilize the receptors, thereby maintaining the active, signaling state. On the other hand, an antagonist antibody that locks receptor arrangement in antiparallel dimers effectively blocks signaling. This model may aid the design of more effective TNF signaling–targeted therapies.

Introduction

Signaling mediated by the tumor necrosis factor (TNF) receptor superfamily (TNFRSF) is essential for immune regulation, cell proliferation, cell death, and morphogenesis (1–3). Because of these essential roles, TNFRSFs and the TNF superfamily (TNFSF) ligands are attractive targets for treating diverse diseases, such as autoimmunity, cancer, infectious diseases, and graft-versus-host disease. Anti-TNF therapies have revolutionized the clinical landscape of autoimmune diseases during the past two decades, and there are numerous TNFRSF agonists in development for cancer (4). However, a still incomplete understanding of the mechanism of TNRSF signaling hampers the development of more effective therapies. For instance, it has only been recently established that TNF receptor II (TNFR2) is an important pathway of regulatory T cell (Treg) expansion or contraction, even in adult human T cells (5–7). Treg are important targets for both cancer and autoimmune therapies, and there are several TNFRSF receptors expressed on Treg and other immune cells to regulate their function. Therefore, understanding TNFRSF signaling is of key importance to developing effective immune therapies for cancer and autoimmunity. Here, we review the literature on the structure-function of TNFRSF signaling and propose a general model for receptor resting state and activation that reconciles decades of research data.

TNFSF Ligands and Their Receptors can be Classified Based on Functional and Structural Characteristics

The TNFSF consists of 19 ligands and 29 receptors in the human (Table 1) (1, 3). Members of the TNFRSF are type I transmembrane proteins that consist of an ectodomain, a transmembrane domain, and an intracellular domain that recruits signal transduction proteins inside the cell. The ectodomains of TNFRSFs are made up of several cysteine-rich domains (CRDs) that form elongated structures. Receptors can be classified into three groups: (i) death receptors (DRs) that interact with members of the TRAF family; and finally, (ii) decoy receptors (DcRs) with no intracellular interacting partners that act as TNFSF ligand inhibitors. In addition to the DRs listed in Table 1, some ligands, such as CD40L, OX40L, glucocorticoid-induced TNF ligand (GITRL), CD27, CD30L, and 4-1BB (CD137L), bind only to a single receptor, but most of the TNFSF ligands bind to more than one receptor, resulting in complex signaling biology.

A trimeric ligand bound to three receptors forms the basic unit of signaling

The structure of the archetypical member of the TNFSF, TNF, is a trimer (8). Each monomer forms a classic “jelly roll” structure composed of beta sheets that define the TNF homology domain (THD). The individual monomers self-assemble into noncovalent homotrimers. The assembled trimers resemble a “cork” that is wider on the top and narrower on the bottom. Despite low to moderate sequence homology, all TNFSF members show high structural homology (2, 3, 8–29). A homotrimer has been observed for all TNFSF members with the exception of dimeric murine GITRL (30) (that likely represents a non-functional state) and the heterotrimeric LTαβ (12). The corresponding receptor monomers bind on the outside at the interface between two ligand monomers (Fig. 1) (17–21, 31–40). This is critically important for receptor activation, as it ensures that only an intact trimeric ligand can trigger signaling because even the loss of a single ligand monomer results in the loss of two receptor-binding sites.

Ligand-free receptors form dimers

The free receptors can form either parallel or antiparallel dimers in solution that likely coexist in equilibrium (Fig. 2) (41). Parallel dimers are formed in a back-to-back fashion, exposing the ligand-binding site. It has been suggested that the role of the parallel dimer in solution is to mop up the free ligands from serum. As an example, soluble TNFR2 (sTNFR2)
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acts as a DcR and immune suppressor by binding and neutralizing sTNF. The anti-TNF drug etanercept (Enbrel) mimics this strategy using an sTNFR2 parallel dimer construct attached to an Fc antibody domain (42). The antiparallel dimers on the other hand bury part of the ligand-binding region and therefore are unable to bind the ligand. It has been proposed that the antiparallel dimers may represent the resting or “nonsignaling” state of the receptor (41), which, when locked in this conformation by an antibody, can efficiently block ligand binding and receptor activation (43). There is further experimental evidence to support the formation of antiparallel dimers as a structure to inhibit receptor signaling. Poxvirus TNFR-like T2 proteins contain a conserved viral preligand assembly domain (PLAD) that is believed to play a role in maintaining the resting state of the receptor (44).

Table 1. TNFRSF receptors, their ligands, and their intracellular binding partners. TNFRSF members can be classified into three groups based on their downstream interaction partners. Some receptors can be activated by more than one ligand. EDARADD, EDAR-associated DD; NADE, neurotrophin-associated cell death executor; NGF, nerve growth factor; NGFR, NGF receptor; n/a, not applicable.

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<th>Intracellular binding partner</th>
<th>TNFSF ligand (TNFSF#, other names)</th>
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<td><strong>Receptors with TRAF-interacting motif (82)</strong></td>
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Fig. 1. Representative structures of TNFSF ligand-receptor complexes and their downstream signaling partners. (A) The tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL)–death receptor 5 (DR5) complex [Protein Data Bank (PDB) ID: 1DU3] represents the structure of a death domain (DD)–interacting TNF superfamily (TNFSF) receptor and its ligand shown in cartoon representation as side (left) and top view (right). The TRAIL ligand trimer is shown in magenta surrounded by three monomers of the DR5 receptor shown in blue. The zinc ion of TRAIL is shown as a gray sphere. (B) Below the cell membrane, the intracellular portion of DR5 contains a DD that can exist in a closed (orange) or open (blue) conformation shown in a schematic representation based on the structure of the FasDD (73). Two helices, helices 5 and 6, are highlighted. Upon activation, the conformational change of helix 6 results in the formation of a long helix that enables DD dimerization and the binding of Fas-associated death domain (FADD) (shown in green). The red circles show the trimerization interface. (C) The TNF–TNF receptor 2 (TNFR2) complex (PDB ID: 3ALQ) illustrates a TNFR-associated factor (TRAF)–interacting TNFR in complex with its ligand. A trimeric TNF ligand (magenta) is bound on the outside by three TNFR2 receptor monomers (blue). In one of the TNFR2 receptor monomers on the far right, the four cysteine-rich domains (CRD1 to CRD4) are labeled and the disulfide bonds are illustrated as yellow spheres. (D) The intracellular portion of TNFR2 (blue line) interacts with TRAF2 shown in chartreuse. TRAF2 consists of three major parts: the TRAF2 C-terminal (TRAF2-C) domain, a long coiled coil (CC) domain, and the TRAF2 N-terminal domain. The structural model of the TRAF2-C–CC complex was created by merging the structures of the TRAF2-C–TNFR2 peptide complex (PDB ID: 1CA9) and the TRAF2 CC domain (PDB ID: 3M06). The black arrow indicates the cellular inhibitor of apoptosis protein 1 or 2 (cIAP1/2)–binding site. The TRAF2 N-terminal domain is made up of four zinc finger domains (ZF1 to ZF4) and a really interesting new gene (RING) domain shown in a schematic representation. Upon activation, TRAF2 trimerizes and activates downstream signaling events, resulting in canonical or noncanonical nuclear factor κB (NF-κB) activation. The program PyMOL was used to create the molecular representations (96).
to form an antiparallel heterodimer with TNFR1 to inhibit TNFR1-driven signaling and cell death (44).

Receptor clustering is required for efficient signaling
For efficient signaling, receptors preassemble on the cell surface. Receptor monomers interact through the so-called PLAD formed by the N-terminal and the CRD1 domains of the receptor (45–49). From the parallel dimer structure, a model of surface arrangement has been proposed showing parallel dimers arranged in a tightly packed hexagonal arrangement with room only for ligand binding (Fig. 2A). This model concurs with the need for PLAD interactions. However, as we discuss in the following sections, this very tight arrangement is inconsistent with more recent findings on TNFRSF and downstream signaling geometries.

We will use two examples to illustrate TNFRSF signaling. In the first example, we will illustrate how TNFRSF members with a DD signal and how these receptors can be targeted by agonistic agents for cancer
immunotherapy. In the second example, we will show how the TRAF-interacting receptor, TNFR2, signals and how blocking this receptor by an antagonist antibody can be used to down-regulate immunosuppressive Tregs and eliminate tumor cells. We will also briefly discuss the structure-function of downstream signaling partners and how these different models can be combined into a unified model of receptor signaling.

**TNFSF Ligands Are Essential for TNFRSF Signaling—Lessons from TRAIL**

TNFSF ligands have diverse biological function including the induction of apoptotic cell death that makes them attractive targets for cancer immunotherapy (4). The TNF-related apoptosis-inducing ligand (TRAIL or Apo2L) is a particularly promising target because it has been shown to selectively induce apoptosis in cancer cells (50, 51). TRAIL signaling biology is very complex because it can bind to four transmembrane receptors: TRAIL receptor 1 (TRAILR1; also known as DR4), TRAILR2 (also known as DR5), TRAILR3 (also known as DcR1), and TRAILR4 (also known as DcR2) and the soluble receptor osteoprotegerin (OPG) (52–56). TRAIL induces apoptosis via its two cognate DRs, TRAILR1 and TRAILR2. The differential expression of TRAIL receptors on different cell types can modulate TRAIL’s effect, resulting in cell death on certain cell types but not on others.

TRAIL, like all TNFSF ligands, shares the classic THD and forms noncovalent homotrimers (Fig. 1A) (31). Ligand trimerization is essential for signaling by TNFSF members. However, the trimers tend to dissociate at low concentrations such as in serum, resulting in their degradation (57). Like other TNFSF ligands, human recombinant TRAIL (hrTRAIL) is less effective at signaling than its transmembrane analog. In particular, hrTRAIL is less efficient at signaling through its receptors than the transmembrane form of TRAIL. TRAIL is unique among the TNFSF ligands in that it contains a single Cys residue (Cys230) per monomer, three of which come together in the active trimer to coordinate a zinc ion (Fig. 1A) (16). Although the zinc ion is strongly bound, it can be lost during purification or in storage. Loss of zinc coordination results in internal disulfide formation between two of the three Cys residues, leading to asymmetry and inactivation (38). Thus, the role of the zinc ion is to stabilize the active TRAIL trimers. Because of its instability, hrTRAIL also has an unfavorable pharmacokinetic profile with a very short half-life of 3 to 5 min and elimination half-life of 20 min (50, 59). To overcome these issues and to improve the cancer targeting potential of hrTRAIL, several different approaches are being pursued (60). These approaches fall into three major categories: (i) efforts to increase trimer stability, (ii) the passive targeting of hrTRAIL to cancer cells, and (iii) the active targeting of hrTRAIL to cancer cells (60). One approach of trimer stabilization is the addition of an external trimerization domain (61) at the N terminus of hrTRAIL such as a leucine zipper motif (LZ-TRAIL) (50), isoleucine zipper motif (iz-TRAIL) (62), or a tenascin-C domain (TNC-TRAIL) (63). The addition of these trimerization domains contributes to much greater stability and improves pharmacokinetic profiles including substantially longer half-life and elimination half-life. As an example, the half-life of iz-TRAIL is 1.3 hours and its elimination half-life is 4.5 hours, several fold higher than that of hrTRAIL. Even more encouraging is that these proteins have shown no specific toxicity on primary human hepatocytes or systemic toxicity in vivo in mice (50, 64). However, none of these improved TRAIL trimers have entered the clinic because the external additions introduce immunogenicity problems that preclude their use in therapeutic applications where repeated dosing is required for efficacy. A more promising approach of trimer stabilization is the production of a single-chain derivative of hrTRAIL that expresses the protein as a single polypeptide chain with small peptide linkers between monomers. This construct has shown improved stability and comparable activity to hrTRAIL (61, 65).

Trials with hrTRAIL or the TRAIL receptor agonist antibodies separately produced disappointing results in the clinic (66–69). However, more recent studies have found that the combination of DR5-specific antibodies and hrTRAIL can synergize to kill cancer cells both in vitro (70, 71) and in vivo (70). This combination therapy proved beneficial even in some previously TRAIL-resistant cancers. The structure of the TRAIL, DR5, and the DR5 agonist antibody illustrates how the coadministration of TRAIL and the agonistic antibody achieves enhanced signaling (70). The antibody binds on the outside of each receptor linking two individual ligand-receptor complexes together into a large hexagonal network (Fig. 3A). Whereas the ligand is essential for receptor activation and signaling, the role of the antibody is to link the individual signaling units together for enhanced signaling. Because of the high structural homology, this model may be applicable to all TNFSF ligands and receptors. As an example, TRAILR2 agonism also shows synergistic activation when both TNF and a cross-linking TNF-R2 agonist antibody are coadministered (43).

Beyond TRAIL receptor targeting antibodies, TNFRSF agonist monotherapies for cancer have uniformly failed to move beyond phase 2 clinical trials. The lessons learned from TRAIL signaling are therefore informative for other TNFSF members as well. In particular, the success of the hrTRAIL/DR5 agonist antibody combination therapy can inform the design of better combination therapies for other TNFRSF members.

**The Characterization of Dominant TNFR2 Antagonist Antibodies Hint at Antiparallel Dimer Assembly and Neutralization on Cell Surface**

TNFR2 represents a member of the TRAF-interacting TNFRSF. It has been recognized as a potent oncprotein and a bidirectional switch for expansion of the immunosuppressive Treg population (7, 43). These dual roles make TNFR2 agonism an attractive approach for autoimmunity and regenerative medicine (72), and TNFR2 antagonism an emerging novel approach for cancer immunotherapy (43). Recessive and dominant antagonists raised against TNFR2 can block TNF binding (43). In this context, we define recessive or dominant antagonists based on how well they can compete with TNF. When TNF was added to the cultures, the TNFR2-directed recessive antibodies competed poorly with TNF. In contrast, dominant antagonists could effectively block TNF signaling even in the presence of increasing concentrations of TNF. Further characterization revealed that recessive antibodies bind in the CRD2 region of TNFR2 and the dominant antagonists bind in the CRD4 region of TNFR2. Furthermore, only the full antibody or the F(ab′)2 structure is able to block TNF. The binding sites of the recessive and dominant TNFR2 antibodies in the TNFR-TNF complex structure all map to the inside of the TNFR2 trimer and would block TNF binding. However, they do not explain the requirement for the obligatory F(ab′)2 structure because a single Fab fragment should be able to displace TNF. This structure also does not explain why the dominant antagonist antibodies preferentially bind in the lower CRD3-CRD4 regions of TNFR2, whereas the recessive antibodies bind in the upper CRD2 portion.
As discussed above, the ligand-free form of TNFRSF members can exist as parallel or antiparallel dimers. The antiparallel dimers are of special interest because they occlude the TNF-binding site (41). To maintain the required threefold symmetry for signaling, the antiparallel receptor dimers might arrange on the cell surface in a hexagonal lattice, where each lattice point brings together three receptor monomers.

Fig. 3. Hexagonal models of extracellular and intracellular signaling networks. (A) Structural representation of the hexagonal network model of a TNFSF ligand–DR–agonist antibody (AgAb) complex (TNFSF-DR–AgAb) based on the TRAIL–DR5–AMG 655 structure (top left; PDB ID: 4N90) (70). In top view, the TNFSF ligand trimers are shown in magenta, surrounded by three receptors shown in blue. Each ligand-receptor complex is cross-linked by an agonist antibody F(ab')2 fragment (green) that stabilizes the hexagonal network. (B) Hexagonal model of the downstream signaling partner of the DR complex shown in (A). The TNFRDD-FADD complex shown in Fig. 1B can cluster to form a hexagonal network (73). (C) Receptor resting state model based on the hexagonal antiparallel dimer network shown in Fig. 2B. (D) TRAFs represented in Fig. 1D can form hexagonal networks via the dimerization of the TRAF N-terminal RING domains. Coloring is the same as in Fig. 1D. All four models in (A) to (D) are built on the same hexagonal lattice indicating a common arrangement for TNFSF DRs, TRAF-associated TNFRs, and their downstream signaling partners.
The dimer is formed by receptor monomers from two different signaling units that occlude the ligand-binding site, maintaining a resting state. This state can most effectively be stabilized by the recently characterized dominant antagonist antibodies that firmly lock in the antiparallel dimer (43). If TNFR2 antagonist antibodies stabilized the antiparallel dimer, this would explain the requirement for at least F(ab')2 structures but failure of a single Fab antibody fragment to confer antagonism (43). In addition, there is no added benefit of adding a cross-linking reagent to either augment antagonism or, worse yet, convert antagonism into agonism. The antiparallel dimeric form of TNFR2 fits best with the accessible binding site for antagonistic antibodies and the functional assays. In the absence of antagonist antibodies, activation by TNF or by other means breaks up the dimer interface and brings the receptors into an upright position, perpendicular to the cell surface, replacing the receptor-receptor interactions with the more favorable receptor-ligand interactions to initiate signaling but preserving the hexagonal symmetry. The model also explains why the recessive antagonist antibodies that bind to the CRD2 region are less effective. The CRD2 regions are too close together in the antiparallel dimer model and can only accommodate one Fab binding; the other Fab would have to bind on a neighboring antiparallel dimer in the hexagonal lattice, a less efficient arrangement that can be more easily disrupted by TNF at high concentrations.

The Downstream Signaling Complexes Form Hexagonal Networks

To better understand the geometric constraints that govern TNFSF signaling, we need to briefly discuss the structure-function of their downstream signaling partners. TNFRSF DRs such as Fas, TNFR1, DR3, DR4, and DR5 interact through their DDs directly or indirectly with the FADD to induce caspase 8–dependent apoptosis. The structure of the Fas DD and FADD (FasDD-FADD) complex (73) provides a template for how DDs in general may interact with FADD to propagate signaling. FasDD, FADD, and the DDs of all TNFRSF members are part of the DD superfamily (DDS) (74, 75). The structure of DDS members consists of six helices and shows high structural homology (76–80). FasDD can exist in a closed or open conformation (Fig. 1B). In the open conformation observed in complex with FADD (73), helix 6 unwinds and, with helix 5, forms a long helix termed the stem helix. The stem helix with the C-terminal helix provides a platform for self-dimerization and the recruitment of FADD. FADD, in turn, recruits caspase 8 through the death effector domain (DED) to initiate clustering and apoptosis. The FasDD-FADD complex bridge is governed by weak protein-protein interactions and proximity. Fas ligand binding initiates the conformational change in FasDD that results in DD dimerization between DDs from neighboring trimers. FasDD also recruits FADD and other downstream signaling partners to form a highly oligomeric structure with hexagonal symmetry (Fig. 3B). This large signaling complex is only assembled after ligand binding provides the on switch for clustering and apoptosis induction. The hexagonal model concurs with the hexagonal model of extracellular DR activation and stabilization seen for TRAIL/DR5 and the DR5 agonist antibody and likely applies to other DRs.

TRAF-interacting receptors such as TNFR2, OX40, CD40, and GITR directly interact with one of six TRAF family members (Table 1) to initiate canonical or noncanonical nuclear factor κB (NF-κB) activation (81). TRAFs contain a C-terminal domain (TRAF-C), a coiled coil (CC) domain, several zinc finger (ZF) domains, and, with the exception of TRAF1, an N-terminal really interesting new gene (RING) domain (Fig. 1D) (82). Research indicates that TRAFs can exist in a monomer-trimer equilibrium depending on their intracellular concentration (83). After TNFSF ligand binding and receptor activation, the intracellular portion of the receptor engages the TRAF-C domain and subsequent TRAF N-terminal RING domain dimerization creates a large signaling network consistent with a hexagonal symmetry (Fig. 3D) (84–86). Tripartite motif proteins that represent the largest subfamily of RING E3 ubiquitin ligases also form a hexagonal lattice structure, indicating that this may be a more general feature of signaling complexes with a RING domain (87–89). In addition to TRAF oligomerization, TRAFs can also bind one or three molecules of cellular inhibitor of apoptosis protein 1 or 2 (cIAP1/2) (90, 91). The number and affinity of cIAP binding to TRAF is controlled by TRAF homotrimers contributing to complex signaling that controls cell fate (82). The conformational change of the extracellular receptor domains upon ligand binding and activation facilitates TRAF direct or indirect recruitment to the intracellular domains of these TNFSF receptors (84, 86). This agonist signal subsequently engages other signaling proteins to activate the inhibitor of κB (IκB) kinase (IKK) and mitogen-activated protein (MAP) kinases, ultimately activating nuclear factor κB (NF-κB). There is still a lot to be learned about TRAF recruitment to the different TNFRSF receptors and their exact arrangement, but it seems clear that intracellular TRAF-associated signaling is also facilitated through a large complex hexagonal network.

TNFRSF Signaling may be Described by a Uniform Hexagonal Model of Receptor Resting State and Activation

We now have experimental evidence for a hexagonal arrangement in the DR5 complex with TRAIL and a DR5 agonist antibody (Fig. 3A) (70), in the intracellular FasDD-FADD complex (Fig. 3B) (73), and for TRAF2 and TRAF6 oligomerization, consistent with a hexagonal lattice geometry (Fig. 3D) (84, 85). We also have evidence of dominant antagonist antibodies blocking TNF binding on the surface of TNFR2 that is best characterized with ligand–free receptors forming antiparallel dimers and arranged in a hexagonal lattice on the cell surface (Fig. 3C) (43). Hexagonal networks are special for several reasons. Regular hexagons are the highest-order polygons that can be tiled or tessellated in a regular pattern by themselves, where each hexagon is surrounded by six other hexagons, repeated indefinitely in any direction with no gaps in between. The best-known example from nature is the honeycomb structure made by bees. Hexagonal tessellation uses the smallest perimeter to enclose a particular area in space (92). Hexagonal structures are also strong. Therefore, the use of a hexagonal network is one of the most efficient ways to build a signaling network by TNFRSF receptors and their downstream partners.

On the basis of the high structural homology within the TNFRSF and the shared downstream signaling partners, we can propose a unified model of TNFRSF resting state and activation (Fig. 4). Trimeric receptors prearrange on the surface. Two monomers from neighboring trimers form antiparallel dimers that exclude the ligand, thus representing the nonsignaling state. The combination of the trimer and dimer symmetries results in the formation of a large hexagonal network.
Fig. 4. TNFSF resting state and activation model. TNFSF receptor monomers (shown in blue) form antiparallel dimers that exclude the ligand, thus representing the resting state. The combination of the trimer and dimer symmetries results in the formation of a hexagonal network represented in top view on the right side. The hexagons can be repeated to form an infinite network. The nonsignaling state of the receptor can be stabilized by antagonist antibodies (shown in yellow) that lock in the antiparallel dimers, and thus block ligand binding and signaling. For dominant inhibition, the full antibody or an F(ab')2 fragment is required. In the absence of a blocking antagonist antibody, TNFSF ligand binding activates the receptors to form signaling complexes that retain the hexagonal symmetry. The TNFSF ligand likely comes into contact with the receptor at the exposed stem region and initiates the conformational change required for ligand binding, as illustrated by the red arrows. The resulting signaling network can be stabilized by cross-linking agonist antibodies shown in green. The protein molecules are shown in surface representation using the same coloring as before.
Receptor activation requires the opening of the antiparallel dimers to a binding competent state. This step is most likely initiated by the ligand itself. It has been shown that the stem (or stalk) region plays an important role in receptor activation (93). This is in good agreement with the antiparallel dimer model of resting state because the antiparallel dimers (but not the parallel dimers) expose the stem region to ligand interactions (Fig. 4). The membrane form of the ligand is more effective at signaling than the soluble ligand because the membrane arranges the ligands in likely the same hexagonal conformation for simultaneous receptor binding and activation. Ligand binding results in the trimerization of the receptor transmembrane domain (94). This, in turn, brings the intracellular portion of the receptors into the right conformation and proximity to enable the successful binding and clustering of the intracellular binding partners to initiate downstream signaling events. The advantage of this model is that it does not require large lateral movement of the receptors in the membrane going from resting state to signaling state, and only a conformational change is required to replace the receptor-receptor interactions with the more favorable receptor-ligand interactions. Arranging the extracellular receptors in a hexagonal geometry makes it easier for the intracellular signaling networks with the same or similar hexagonal geometry to form.

One of the important questions in TNFSF signaling is how accidental signaling is prevented if the receptors are prearranged on the surface. Our model provides an explanation of how this can be achieved. The antiparallel dimers bury the ligand-binding site and put the receptors in a nonsignaling state. Receptor activation in the absence of ligand is much less likely in the antiparallel dimer than in the parallel dimer model. The conformational change initiated by the ligand causes further conformational changes in the transmembrane and intracellular regions of the receptor that enable the binding of downstream signaling partners. We also discussed above how dominant antibodies can successfully block the binding of the ligand by firmly locking in the antiparallel dimer state of the receptor. Once activated, however, receptor trimers can be stabilized by cross-linking agonist antibodies (70). The antibodies are unable to activate the receptor; their role is to provide structural stabilization to the active signaling network initiated by ligand binding. Receptor cross-linking antibodies may also block the access of enzymes whose role is to cleave off the receptor. In this way, cross-linking antibodies can maintain the signaling competent state longer. None of this could be achieved in the parallel dimer model that places the receptor trimers tightly together with no room for antibody binding (41). The parallel dimer model is inconsistent with the need of cross-linking agonist antibodies and their signaling complexes (70) or with the proposed geometry of intracellular signaling networks that engage TNFRSF receptors (73, 84, 86). These more recent findings are all consistent with the hexagonal network model that places the individual ligand-receptor complexes ~120 Å apart with a total edge length of ~170 Å (the number may vary with receptor type).

**Concluding Remarks**

Successful TNFRSF activation requires the corresponding TNFSF ligand; agonist antibodies alone are unable to activate signaling effectively. This explains the relatively modest results in the clinic by agonist antibody monotherapies. More efficient activation can be achieved by the ligand or coadministration of a stable trimeric ligand and a receptor cross-linking antibody. Alternatively, a ligand analog with membrane-like features can be used as well. The picture that emerges from the literature indicates that the membrane serves at least two functions: (i) it anchors the trimeric TNFSF ligands and increases their stability and bioavailability, and (ii) it provides a platform for the arrangement of multiple ligand trimers for simultaneous receptor activation and enhanced signaling. These findings have created renewed interest in the production of stable TNFSF ligand analogs with increased activity, longer half-life, and low immunogenicity (60, 95). Future designs will need to incorporate the hexagonal model of activation to design more effective ligand analogs or to use stable trimeric ligands and receptor cross-linking antibody combination therapies. On the other hand, efficient TNFRSF antagonism requires locking in the antiparallel dimers that represent the resting state of the receptor preassembled on the cell surface. It has been challenging to create truly dominant antagonist antibodies, but with these new insights into the resting state geometry, it will be possible to design more efficient antagonists to TNFRSF receptors. The hexagonal model that incorporates both extracellular and intracellular signaling geometries provides a compelling model of TNFRSF resting state and activation. The antiparallel dimer conformation satisfies the requirement for receptor preassembly on the surface of the cell via the PLAD. Arranging receptor ectodomains in the right hexagonal geometry on the cell surface makes it easier to assemble the intracellular hexagonal signaling complexes as well. This is crucial because the protein–protein interactions that govern the formation of the intracellular signaling complexes are weak and depend on the interacting partners to be in close proximity and in a specific conformation. The resting state and activation model should inform the future design of better receptor-specific agonists and antagonists for the whole TNFRSF.

**REFERENCES AND NOTES**


31. J. M. Lesslauer, Crystal structure of human RANKL complexed with its decoy receptor osteoprotegerin.


37. J. Wallweber, D. M. Compaan, M. A. Starovasnik, S. G. Hymowitz, The crystal structure of human RANKL complexed with its decoy receptor osteoprotegerin.

38. J. Wallweber, D. M. Compaan, M. A. Starovasnik, S. G. Hymowitz, The crystal structure of extracellular human BAFF, a TNF family member that binds to TACI and APRIL-receptor complexes: Like BCMA, TACI employs only a single cysteine-rich domain.

39. J. Wallweber, D. M. Compaan, M. A. Starovasnik, S. G. Hymowitz, The crystal structure of extracellular human BAFF, a TNF family member that binds to TACI and APRIL-receptor complexes: Like BCMA, TACI employs only a single cysteine-rich domain.


76. The PyMOL Molecular Graphics System, Version 1.7 (Schrodinger, LLC).

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