Review

Principles of antibody-mediated TNF receptor activation

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From the beginning of research on receptors of the tumor necrosis factor (TNF) receptor superfamily (TNFRSF), agonistic antibodies have been used to stimulate TNFRSF receptors in vitro and in vivo. Indeed, CD95, one of the first cloned TNFRSF receptors, was solely identified as the target of cell death-inducing antibodies. Early on, it became evident from in vitro studies that valency and Fcγ receptor (FcγR) binding of antibodies targeting TNFRSF receptors can be of crucial relevance for agonistic activity. TNFRSF receptor-specific antibodies of the IgM subclass and secondary cross-linked or aggregation prone dimeric antibodies typically display superior agonistic activity compared with dimeric antibodies. Likewise, anchoring of antibodies to cell surface-expressed FcγRs potentiate their ability to trigger TNFRSF receptor signaling. However, only recently has the relevance of oligomerization and FcγR binding for the in vivo activity of antibody-induced TNFRSF receptor activation been straightforwardly demonstrated in vivo. This review discusses the crucial role of oligomerization and/or FcγR binding for antibody-mediated TNFRSF receptor stimulation in light of current models of TNFRSF receptor activation and especially the overwhelming relevance of these issues for the rational development of therapeutic TNFRSF receptor-targeting antibodies.

Facts

- Ligands of the TNF superfamily (TNFSF) occur as trimeric transmembrane proteins but also as soluble trimeric molecules.
- A subgroup of the TNF receptor superfamily (TNFRSF) is not or only slightly activated by soluble TNFSF ligands.
- Oligomerization and cell surface-anchoring of soluble TNFSF ligands provide these molecules with membrane TNFSF ligand-like activities.
- Dimeric TNFRSF receptor-specific antibodies have typically no or only a moderate agonistic activity.
- Oligomerization and Fcγ receptor-binding frequently converts dimeric TNFRSF receptor-specific antibodies into strong agonists.

Open Questions

- What are the mechanisms underlying the FcγR binding-independent agonistic activity of TNFRSF receptor-specific human IgG2 isoform B antibodies?

General Principles of TNFRSF Receptor Activation by Ligands of the TNF Superfamily

Receptors of the tumor necrosis factor (TNF) receptor superfamily (TNFRSF) are naturally activated by ligands of the TNF superfamily.1,2 Cytokines are assigned to the TNF superfamily (TNFSF) based on a conserved carboxy-terminal homology domain called the TNF homology domain (THD) (Figure 1).1,2 The THD promotes the assembly of homotrimeric molecules, or in rare cases the formation of dimeric (murine GITRL)3,4 or heterotrimeric (LTαβ2)5 ligands, and is essential for interaction with receptors of the TNFRSF. With exception of LTα, TNFSF ligands are expressed as trimeric type II transmembrane proteins in which the THD is separated from the transmembrane domain by a stalk region of variable length (Figure 1). Due to proteolytic processing in the stalk region or by alternative splicing, TNFSF ligands can also be found in the form of soluble trimeric molecules (Figure 1). Soluble TNFSF ligands still contain the THD and thus retain the ability to interact with TNFRSF receptors.1,2 X-ray crystallographic studies of various soluble TNFSF ligands, alone or in complex

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Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; cIAP1/2, cellular inhibitor of apoptosis; FcγR, Fcγ receptor; Fn14, fibroblast growth factor inducible; NfκB, nuclear factor κB; NIK, NfκB inducing kinase; PLAD, pre-ligand assembly domain; TACI, transmembrane activator and CAML interactor; TNFR1, TNF receptor-1; TNFRSF, tumor necrosis factor (TNF) receptor superfamily; TRAF2, TNF receptor associated factor-2; TRAIL, TNF-related apoptosis inducing ligand; TWEAK, (TNF)-like weak inducer of apoptosis.

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with TNFRSF receptor ectodomains (Table 1), not only confirmed the trimeric organization of TNFSF ligands deduced from biochemical assays but also revealed that each of the three protomer–protomer interfaces of a TNFSF ligand trimer binds a single TNFRSF receptor molecule.

In view of the structural organization of TNFSF ligand/TNFRSF receptor complexes, a sequential model of TNFRSF receptor activation was initially assumed. According to this model, a single TNFRSF receptor molecule initially interacts with a TNFSF trimer and the resulting cell surface-associated TNFSF ligand–TNFRSF receptor complex then recruits in two further steps two additional monomeric TNFSF receptor molecules to form an active TNFSF ligand–TNFRSF receptor 3 complex (Figure 2a). This early model of TNFRSF receptor activation, however, is incompatible with some fundamental observations. First, ligand binding studies gave no evidence for a sequential assembly of TNFSF ligand–TNFRSF receptor complexes and consistently argued for a single binding site interaction between TNFSF ligands and TNFRSF receptors. Second, the affinity of a single soluble TNFRSF receptor ectodomain for its ligand is usually rather low (\( > 1 \mu M \))\(^{6,7} \). Indeed, efficient functional neutralization of TNFSF ligands with soluble TNFRSF receptor variants requires the assembly of two or more receptor molecules, for example, by genetic fusion with dimerizing or trimerizing protein domains (e.g., Holler et al.\(^{8} \)). Third, the sequential TNFRSF receptor activation model cannot explain why some mutants of the TNFRSF receptors CD95 and TACI, which are defective in ligand binding, nevertheless act in a dominant-negative manner and cause autoimmune lymphoproliferative syndrome (ALPS)\(^{9} \) and common variable immunodeficiency (CVID).\(^{10} \)

The limitations of the sequential TNFRSF receptor activation model were solved by the discovery of a protein domain...
| Structure | PDB ID | Resolution (Å) | Ref. |
|-----------|--------|----------------|------|
| Human TNFR1-LTα | 1TNR | 2.85 | 99 |
| Human TNF | 1TNF | 2.6 | 100 |
| Human LTα | 1.9 | 101 |
| Human TNFR1 | 1EXT | 1.85 | 102 |
| | 1NCF | | 103 |
| Human TNFR2 | 3ALQ | 3 | 104 |
| Human LTαββ2-LTβR | 4MXW | 3.6 | 105 |
| Human CD40L | 1ALY | 2 | 106 |
| Human CD40L-CD40 | 3QD6 | 3.5 | 107 |
| Murine OX40L | 2HEW | 1.45 | 108 |
| Murine OX40L-humanOX40 | | 2 | 108 |
| Human OX40L-humanOX40 | 2HEV | 2.41 | 108 |
| Human 4-1BB | 2X39 | 2.3 | 109 |
| Human TRAIL | 1DG6 | 1.3 | 110 |
| | 1D2Q | 2.8 | 111 |
| Human TRAILR2-TRAIL | 1DU3 | 2.2 | 112 |
| | 1D0G | 2.4 | 113 |
| | 1DV1 | 2.2 | 114 |
| Murine RANKL | 1J7Z | 2.6 | 115 |
| | 1SSS | 1.9 | not recorded in Pubmed |
| | 1IQA | 2.2 | 116 |
| Murine RANK | 3M64 | 2.01 | 117 |
| Human RANKL-OPG | 3URF | 2.7 | 118 |
| Murine RANKL-RANK | 3BQG | 2.5 | 119 |
| | 4GIQ | 2.7 | 7 |
| | 3ML2 | 2.8 | 117 |
| Murine RANKL-OPG | 4E4D | 2.7 | 7 |
| Murine GITRL | 2Q8O | 1.75 | 4 |
| | 3BC9, 2QDN | 1.76 | 120 |
| Human GITRL | 2R32, 2Q1M | 1.95, 2.3 | 121 |
| Human TL1A | 2QF3 | 2.5 | 122 |
| | 2RE9, 2000 | 2.1, 3 | 123 |
| Human DcR3 | 3MHD | 2.9 | 124 |
| Human TL1A-DcR3 | 3MIB, 3K51 | 2.95, 2.45 | 124 |
| Human CD95L-DcR3 | 4MSV | 2.5 | not recorded in Pubmed |
| Human LIGHT-DcR3 | 4J6G | 2.4 | 125 |
| Human LIGHT | 4EN0 | 2.59 | 125 |
| Human LIGHT-HVEM | 4RSU | 2.3 | not recorded in Pubmed |
| Human APRIL | 1USZ | 2.4 | 126 |
| | 1USY | 2.3 | 126 |
| | 1USX | 1.8 | 126 |
| Human Baff | 1JHS | 3.0 | 127 |
| | 1KD7 | 2.8 | 128 |
| | 1KXG | 2 | 129 |
| Human BaffR | 1OSX | Solution NMR | 130 |
| Human TACI-CRD2 | 1XUT | Solution NMR | 131 |
| Human BCMA | 2KN1 | Solution NMR | 132 |
| Human Baff-BCMA | 1OQE | 2.5 | 133 |
| Human Baff-BCMA | | | 134 |
| | 1OTZ, 1P0T | 3.3 | 134 |
| Human APRIL-TAC1 | 1UX1 | 1.9 | 131 |
| Human APRIL-BCMA | 1UX2 | 2.35 | 131 |
| Human Fn14 | 2KMQ | Solution NMR | 132 |
| | 2RPJ | Solution NMR | 135 |
| Xenopus Fn14 | 2KN0 | Solution NMR | 132 |
| Human EDA-A1 | 1RJ7 | 2.3 | 136 |
| Human EDA-A2 | 1RS8 | 2.23 | 136 |
| Human DR6 | 3Q04 | 2.2 | 137 |
| | 3U3V | 2.96 | 138 |
| | 3U3T | 3.21 | 138 |
| | 3U3S | 2.7 | 138 |
| | 3U3Q | 2.7 | 138 |
| | 3U3P | 2.09 | 138 |
| Rat NT3-NGFRp75 | 3BUK | 2.6 | 139 |
| Rat NGF-NGFRp75 | 3UJ2 | 3.75 | 140 |
| | 1SG1 | 2.4 | 141 |
within several TNFRSF receptors that mediates self-assembly in the absence of ligand. The self-affinity of TNFRSF receptors would not only allow to explain TNFSF ligand binding by formation of high affinity dimeric or trimeric TNFRSF complexes but may also drive secondary interaction of TNFSF ligand–TNFRSF receptor₂ complexes. The initially formed TNFSF ligand–TNFRSF receptor₂ complexes may already allow the recruitment of TNFRSF receptor-associated signaling molecules but do not ensure full activation of these molecules by transactivation. Please note, the capacity of soluble TNFSF ligand-induced TNFSF ligand–TNFRSF receptor₂ complexes to secondary aggregate spontaneously into fully active receptor clusters may vary considerably between TNFRSF receptors. In some cases (right, upper part) the self-affinity of TNFRSF receptors is maybe too low to trigger spontaneous clustering of soluble TNFSF ligand-induced receptor complexes while in other cases (right, lower part) the self-affinity is high enough to trigger this

Figure 2 PLAD-assisted oligomerization model of TNFRSF receptor activation. This model is based on the fundamental observation that at least some TNFRSF receptors pre-assembles in the absence of ligand. The self-affinity of TNFRSF receptors would not only allow to explain TNFSF ligand binding by formation of high affinity dimeric or trimeric TNFRSF complexes but may also drive secondary interaction of TNFSF ligand–TNFRSF receptor₂ complexes. The initially formed TNFSF ligand–TNFRSF receptor₂ complexes may already allow the recruitment of TNFRSF receptor-associated signaling molecules but do not ensure full activation of these molecules by transactivation. Please note, the capacity of soluble TNFSF ligand-induced TNFSF ligand–TNFRSF receptor₂ complexes to secondary aggregate spontaneously into fully active receptor clusters may vary considerably between TNFRSF receptors. In some cases (right, upper part) the self-affinity of TNFRSF receptors is maybe too low to trigger spontaneous clustering of soluble TNFSF ligand-induced receptor complexes while in other cases (right, lower part) the self-affinity is high enough to trigger this
molecules would become accessible for ligand binding via the ligand-free PLAD-assembled TNFRSF receptors despite the rare occurrence of this receptor species. Currently, it is not possible to differentiate between the two extremes and there are certainly TNFRSF receptor type-dependent quantitative differences in the PLAD–PLAD interaction that may considerably affect the dynamic equilibrium between monomeric and PLAD-assembled TNFRSF receptors.

The PLAD-based model for the formation of TNFSF ligand3–TNFRSF receptor3 complexes alone, however, does not adequately explain one fundamental observation of overwhelming functional importance namely why a significant fraction of TNFRSF receptors bind soluble TNFSF ligands with high affinity but nevertheless fail to efficiently activate receptor-associated signaling pathways. While interaction with a membrane-bound TNFSF ligand in any case results in strong receptor activation, TNFRSF receptors differ in their response to binding of soluble ligand trimers. Some TNFRSF receptors strongly stimulate intracellular signaling pathways in response to soluble TNFSF ligands whereas another group of TNFRSF receptors binds soluble ligand molecules with a limited effect on signal transduction (Table 2). The limited responsiveness to soluble TNFSF ligands of this second type of TNFRSF receptors reflects an intrinsic quality of the TNFRSF receptor type and not an insufficiency of the soluble ligand. For example, soluble TNF efficiently stimulates TNFR1 signaling but fails to properly activate TNFR2 despite efficient binding.15,16 Similarly, soluble APRIL interacts with the TNFRSF receptors TAC1 and Baff receptor-3 (BR3) but only activates the latter17,18 TNFRSF receptors that fail to signal properly in response to binding of soluble ligand trimers, typically respond quite well when the ligand molecules become secondarily oligomerized (Table 2). The latter can be achieved for example by antibodies recognizing a tag attached to the cytokine molecules or by genetic fusion with protein domains triggering the assembly of two or more ligand trimers in a single molecule (Table 3). Because oligomerization has no major effect on the apparent affinity of TNFSF ligand–TNFRSF receptor interaction,19,20 this indicates that secondary interaction of two or more TNFSF ligand3–TNFRSF receptor3 complexes is a key event in stimulation of TNFRSF receptor-associated signaling pathways.

There is, however, initial evidence that different types of TNFRSF receptor-associated signaling pathways differ in the need for secondary interaction of two or more TNFSF ligand3–TNFRSF receptor3 complexes for activation. The need for clustering of TNFSF ligand3–TNFRSF receptor3 complexes for receptor activation has been typically observed in experiments where apoptosis induction or activation of the classical NFκB pathway has been investigated (see Table 2). Recent studies indicated that soluble CD95L, at low concentrations where it typically fails to trigger apoptosis without crosslinking, induces cell migration and proliferation (for review, see Wajant21). Soluble TWEAK ((TNF)-like weak inducer of apoptosis) furthermore stimulates strong and efficient activation of the alternative NFκB pathway but activates the classical NFκB pathway only weakly whereas both NFκB pathways were strongly activated by membrane TWEAK and oligomerized soluble TWEAK.22 The different oligomerization requirement for CD95L-induced apoptosis and CD95L-induced cell migration as well as the different need of oligomerization for soluble TWEAK-triggered classical and alternative NFκB signaling correspond in both cases to different mechanisms how these pathways are activated. Interestingly, form studies comparing ligand- and antibody-induced activation of CD40 and Fn14, there is also evidence for pathway-specific activation requirements of TNFRSF receptors. For example, it has been reported that antibody production and IL6 secretion in B cells are induced after CD40 stimulation with membrane-bound CD40L while an agonistic CD40-specific antibody triggered antibody but not IL6 production.23 Fn14 targeting antibodies, furthermore, can stimulate the alternative NFκB pathway without a significant effect on the classical NFκB pathway.24

Fn14-mediated activation of the classical NFκB pathway requires the recruitment of the adapter protein TRAF2 and the TRAF2-interacting E3 ligases CIAP1 and CIAP2.25,26 TRAF2 forms homotrimmers that binds tightly to a probably monomeric and thus inactive CIAP1 or CIAP2 E3 ligase molecule.27–30 Dimerization of two CIAPs results in an active form which can promote signaling via the classical NFκB pathway.27,31 Thus, in view of the data discussed above soluble TWEAK seems to induce the formation of complexes that only contain a single CIAP1/2 molecule (TWEAK3-Fn14–TRAF2–CIAP1/2) and which are still unable to trigger the classical NFκB pathway but are competent to do this upon CIAP1/2 transactivation-enabling crosslinking. In contrast, the formation of TWEAK-Fn14 complexes containing only one TRAF2 trimer and a single CIAP1/2 molecule is already sufficient to activate the alternative NFκB pathway, because in this case, it is sufficient to withdraw TRAF2–CIAP1/2 complexes from the cytosolic22,32,33 where they are involved in triggering the destruction of the alternative NFκB inducing kinase NIK. In the case of CD95-
induced apoptosis, there is crystallographic evidence that a pentameric/oligomeric complex of the CD95-recruited death domain-containing adapter protein FADD has to be formed to trigger efficient dimerization and activation of caspase-8 in oligomeric structures.34–38 In contrast, soluble CD95L-induced CD95-mediated cell migration and proliferation are independent from FADD and occur by help of tyrosine kinases that directly interact with CD95.39 In this case, signaling pathway activation could already emerge from CD95L–CD95 complexes. In sum, the evidence for oligomerization-independent selective activation of only certain receptor-associated signaling pathways by soluble TWEAK and soluble CD95L favors a two-step model of TNFRSF receptor activation. In a first step, there is ligand-induced formation of signaling competent TNFSF ligand–TNFRSF receptor complexes, which might already trigger certain signaling pathways. In a second step, there is then oligomerization of TNFSF ligand–TNFRSF receptor complexes that eventually enables activation of signaling pathways requiring transactivation/oligomerization of TNFSF ligand–TNFRSF receptor complex-associated signaling intermediates (Figure 2b).

The capacity of membrane-bound TNFSF ligands to trigger TNFRSF receptor clustering has not been extensively investigated. The finding that membrane-bound CD95L but not soluble CD95L induces the formation of durable supra-molecular ligand-receptor clusters, however, is in good accordance with this idea.40 In accordance with the evidence discussed above that activation of only a subset of CD95-induced signaling pathways, including apoptosis induction, requires oligomerization of CD95L–CD95 complexes and thus membrane-bound CD95L, O’Reilly et al. reported that mice expressing only soluble CD95L have defective CD95-induced apoptosis but also obtained evidence for soluble CD95L-mediated non-apoptotic activities.41 It is furthermore worth mentioning that artificially anchoring soluble TNFSF ligands to the cell surface is all that is required to equip these molecules with the activity of the corresponding membrane-bound cytokine. For example, soluble TNFSF ligand fusion proteins with interaction domains recognizing a cell surface exposed molecular structure/protein acquire membrane ligand-like activity after target binding.50,42,43 Similarly, soluble CD95L gain high apoptotic activity after fibronectin binding and APRIL stimulates Baff-R when trapped by the extracellular matrix via a heparan sulfate proteoglycan binding motif in the stalk region.18,44,45 Moreover, it has been observed that the enhanced TNFR2-stimulating activity of a cell surface-anchored fusion protein of soluble TNF is accompanied by clustering of TNFR2 complexes.46

The two-step model of TNFRSF receptor activation is based on data of the subgroup of TNFRSF receptors that do not or only poorly activate apoptosis and classical NFκB signaling in response to binding of soluble TNFSF ligands. An obvious question that has not been addressed so far is how TNFRSF receptors that are readily activated by soluble TNFSF ligands, such as TNFR1, fit in the two-step model of TNFRSF activation. One possibility is that the PLAD-dependent self-affinity of these TNFRSF receptors is simply high enough to drive secondary clustering of initially formed TNFSF ligand–TNFRSF receptor complexes. However, it cannot be ruled out that this TNFRSF receptor type uses still unknown

### Table 3 TNFSF ligand fusion protein molecules containing two or more TNF trimers

| TNFSF fusion protein | Number of TNF trimers | Examples | EC50 (μg/mL) |
|----------------------|-----------------------|----------|--------------|
| Fc-TNFSF            | 2                     | CD95L    | 1000         |
|                     |                       | OX40L    | ~10          |
|                     |                       | TWEAK    | >100         |
| ACRP-TNFSF          | 2                     | CD95L    | 100          |
|                     |                       | CD40L    | >100         |
| Fe-scTNFSF          | 2                     | TRAIL    | >100         |
| EDH2-scTNFSF        | 2                     | TRAIL    | >10 - 100    |
| TNC-scTNFSF         | 3                     | TNF      | inactive     |
|                     |                       |          | versus highly |
|                     |                       |          | active       |
| Fe-TNC-TNFSF        | 2                     | 4-IBBL   | ~100         |
| SP-D-TNFSF          | 4                     | CD40L    | Improved     |
|                     |                       | BaF      | max. responses |
|                     |                       | 4-IBBL   | highly active |
|                     |                       | OX40L    | highly active |
| Fe-ILZ-TNFSF        | 2                     | OX40L    | highly active |

Abbreviations: ACRP, adiponectin collagen domain; EDH2, immunoglobulin E heavy-chain domain 2; Fc, constant IgG1 domain; ILZ, trimerizing isoleucine zipper domain; scTNFSF, three THD domains connected by peptide linkers; SP-D, surfactant protein D scaffold; TNC, tenascin-C

*The enhancing effect observed in this study depends on the TWEAK-induced pathway considered. Fc-TWEEK showed a 100-fold lower EC50 for classical NFκB signaling compared with Flag-TWEAK while both molecules were equally effective in triggering p100 processing

*Soluble TNFSF ligand trimers have not been analyzed
mechanisms/factors enabling these receptors to promote oligomerization of TNFRSF-associated adapter proteins without oligomerization of TNFSF ligand–TNFRSF receptor complexes.

Relevance of Isotype and Oligomerization for Agonistic Activity of TNFRSF Receptor-Specific Antibodies

Agonistic receptor-specific antibodies were important tools for studying functions of TNFRSF receptors as long as their corresponding TNFSF ligands were unknown and are accordingly still of special relevance for the analysis of the orphan TNFRSF receptors DR6, TROY and RELT. Agonistic antibodies are also a great help for research on TNFRSF receptors that share a common TNFSF ligand, as for example the TNF-related apoptosis inducing ligand (TRAIL) receptors. Above all, however, agonistic antibodies are still the means of choice in scenarios where activation of TNFRSF receptors is needed. Indeed, antibodies have superior pharmacokinetics compared with recombinant TNFSF ligands that have quite low serum half-life of around 10–30 min and therefore require elaborate clinical treatment regimes, such as infusion. Moreover, there is broad experience in the development, production and approval of antibodies. Accordingly, there are various agonistic TNFRSF receptor-specific antibodies that are currently under consideration in clinical trials (Table 4).

Typically, TNFRSF receptor-specific antibodies are used with the intention to activate TNFRSF receptors on tumor cells to trigger cell death (TRAILR1, TRAILR2) or to activate costimulatory receptors on immune cells to promote antitumor immunity (4-1BB, GITR, CD27, OX40 CD40). In some cases (CD30, Fn14), the tumor-associated expression pattern of certain TNFRSF receptors is exploited to target tumor cells with ADCC-inducing antibodies or antibody immunotoxins.

Soon after the description of the first TNFRSF receptor-specific agonistic antibodies, it turned out that the valency of antibodies, thus the antigen binding sites per molecule, is of crucial relevance for the agonistic activity. In a panel of 17 human TNFR1-specific IgG2a and IgG2b antibodies, Engelmann et al. identified only two antibodies that moderately mimicked the cytotoxic activity of TNF while all of the these antibodies showed strong TNFR1-mediated killing upon cross-linking with secondary antibodies. Likewise, it was found that cross-linking converts the antagonistic TNFR1-specific IgG2a antibody H398 into a potent TNFR1 agonist.

Another study characterized the in vitro activities of two IgG1 antibodies and an IgM specific for TNFR1 and reported superior agonistic activity for the pentameric IgM variant. Related data have been reported for CD95-specific antibodies. The highly agonistic CD95-specific antibody APO-1 is an IgG3 and has thus a considerable tendency to self-aggregate. In contrast, IgG1, IgG2a, IgG2b and IgA variants of APO-1, that have no or only a low capacity to aggregate, elicit no or less efficient CD95 activation in vitro. Cross-linking with protein A or secondary antibodies, however, restored the high agonistic activity of these APO-1 variants.

In line with this, various other CD95-specific mAbs of the IgG1 and IgG2a/b subclass have been described that only display strong agonistic activity after cross-linking while the pentameric CD95-specific IgM CH-11, but not Fab2-fragments derived of this antibody, has high, aggregation-independent agonistic activity. The potentiating, or even uncovering, effect of cross-linking on the agonistic activity of dimeric antibodies has also been broadly documented for other TNFRSF receptors including CD40, TRAILR1/DR4, TRAILR2/DR5 and Fn14. The relevance of cross-linking for the agonistic activity of dimeric TNFRSF receptor-specific antibodies is also reflected by the fact that antibodies recognizing non-overlapping epitopes synergistically induce receptor activation. In a variation of this theme, it has been recently demonstrated that the therapeutic agonistic activity of the rat IgG2a murine 4-1BB-specific antibody 3H3 in mouse models of experimental autoimmune encephalomyelitis and allergic asthma is based on the expression of galectin-9 which binds to 4-1BB without affecting antibody binding. Thus, the endogenously present galectin-9 molecule may act as a natural crosslinker here. Although antibody-specific factors, such as affinity and epitope localization in the targeted TNFRSF receptor, certainly play a role for agonistic activity, the data discussed, in sum suggest that the valency of TNFRSF receptor-specific antibodies and antibody preparations is the dominant factor that determines their receptor-stimulatory capacity. In particular in view of the importance of clustering of trimeric ligand–receptor complexes for the activation of TNFRSF receptor-associated signaling pathways, it seems natural that interaction of two or more receptor–antibody complexes is required to form active [receptor–antibody]n aggregates (Figure 3a).

The need for secondary interaction of initially formed trimeric ligand–receptor complexes for full TNFRSF receptor activation is nicely reflected by the ability of some per se non-agonistic TNFRSF receptor-specific antibodies to synergistically stimulate receptor signaling in concert with soluble TNFSF ligands. Already in the 1990s, we described the TNFR2-specific monoclonal antibody 80M2 that allowed robust TNFR2 activation by soluble TNF which alone is an inefficient stimulator of TNFR2 signaling. Likewise, it has been found that poorly active, soluble CD95L trimers synergistically induce cell death with non-apoptotic CD95-specific antibodies and that some CD40-specific antibodies enhance soluble CD40L activity. Of course, a straightforward explanation of these observations is that these TNFRSF receptor antibodies bring together individually assembled trimeric ligand–receptor complexes.

The typically quite limited agonistic potential of bivalent TNFRSF receptor-specific antibodies may further suggest that monomeric receptors are the dominant receptor species in the equilibrium of monomeric receptors and PLAD-assembled receptors. In the case of a significant fraction of PLAD-assembled receptors, one would predict the formation of flexible ‘chains’ or clusters formed due to the bivalency of the antibodies and the two or three epitopes present in dimeric (or trimeric) PLAD-assembled receptors. It is not so obvious why further cross-linking should have here the huge functional relevance that has been observed experimentally. In the case of a low degree of PLAD-driven complex formation, however, cross-linking of dimeric antibodies would have an almost obligate impact on the secondary interaction of receptor–
| Antibody | Target | Isotype | Status | ID | Condition |
|----------|--------|---------|--------|----|-----------|
| Brentuximab-Vedotin SGN-35 | CD30 | Drug conjugate, chimerized IgG1 | Approved, > 70 studies | — | Lymphoma |
| XmAb2513 | CD30 | IgG1 Enhanced FcyR binding | Phase 1 | Completed | NCT00606645 | Hodgkin lymphoma |
| MDX-1401 | CD30 | IgG1 | Phase 1 | Completed | NCT00634542 | Hodgkin lymphoma |
| HeFi-1 | CD30 | Murine IgG1 Agonist | Phase 1 | Completed | NCT00048880 | Neoplasms |
| PF-05082566 4-1BB | IgG2 | Phase 1 | Recruiting | NCT02179918 | Advanced solid tumors |
| Urelumab BMS-663513 | IgG4 | Phase 1 | Recruiting | NCT01775631 | B-cell malignancies |
| TRX518 | GITR | IgG1 N297 Fc-disabled | Phase 1 | Recruiting | NCT01239134 | Stage III/IV melanoma |
| MK-4166 | GITR | Phase 1 | Recruiting | NCT02132754 | Solid tumors |
| Varilumab CDX-1127 | IgG1 | Phase 1 | Recruiting | NCT01460134 | Solid tumors, B-cell NHL |
| MEDI6469 | QX40 | Murine IgG1 | Phase 1 | Unknown | NCT01644968 | Advanced cancer |
| MEDI0562 | QX40 | IgG1 humanized Agonist | Phase 1 | Recruiting | NCT02318934 | Solid tumors |
| CP-870,893 | IgG2 | Phase 1 | Completed | NCT0103635 | Recurrent/IV melanoma |
| PG102 FFP104 | IgG4 | Termination (poor recruitment) | NCT00787137 | Psoriatic arthritis |
| Lucatumumab HCD122 | IgG1 | Phase 2 | Completed | NCT00231166 | Multiple myeloma |
| Chi Lob 7/4 | IgG1 chimeric Agonist | Phase 1 | Terminated | NCT0010108 | CLL |
| ASKP1240 | IgG4 | Phase 1 | Completed | NCT01555681 | Healthy volunteers |
| Enavatuzumab PDL192 | IgG1 humanized | Phase 1 | Completed | NCT00738764 | Advanced solid tumors |
| Conatumumab AMG655 TRAILR2/DR5 | IgG1 | Phase 1b | Completed | NCT00791011 | Lymphoma |
| Lexatumumab HGS-ETR2 TRAILR2/DR5 | IgG1 | Phase 1 | Completed | NCT00428272 | Sarcoma neuroblastoma |
antibody complexes and thus on receptor–antibody chain/cluster formation.

The overwhelming importance of the intrinsically limited activity of soluble TNFSF ligand trimers and dimeric anti-TNFRSF receptor antibodies for the development of TNFRSF receptor-targeting therapeutic concepts becomes particularly apparent in the development of TRAIL death receptor-targeting drugs. TRAIL has been initially identified due to its homologies to TNF. TRAIL binds to five different receptor types that all belong to the TNFRSF receptor family: TRAILR1 to TRAILR4 and osteoprotegerin (OPG). While TRAILR3, TRAILR4 and OPG act as membrane-associated or soluble

| Antibody       | Target               | Isotype | Status    | ID          | Condition      |
|----------------|----------------------|---------|-----------|-------------|----------------|
| Mapatumumab    | TRAILR1/DR4          | IgG1    | Phase 2   | NCT00092924 | NSCLC          |
|                |                      |         | Completed | NCT00094848 | NHL            |
| Tigatuzumab    | TRAILR2/DR5          | IgG1    | Phase 1   | NCT01220999 | CRC neoplasms  |
| CS-1008        |                      |         | Completed | NCT01307891 | Breast cancer  |
|                |                      |         | Terminated| NCT00969033 | Metastatic CRC |
|                |                      |         | Completed | NCT00991796 | NSCLC          |
|                |                      |         | Completed | NCT00521404 | Pancreatic cancer |
|                |                      |         | Completed | NCT00945191 | OC             |
|                |                      |         | Completed | NCT01124630 | Metastatic CRC |
|                |                      |         | Ongoing   | NCT01033240 | Liver cancer   |
|                |                      |         | Completed | NCT00320827 | Malignancies, lymphoma |
| Drozitumab     | TRAILR2/DR5          | IgG1    | Phase 2   | NCT00543712 | Chondrosarcoma |
| PRO95780       |                      |         | Terminated| NCT00480831 | NSCLC          |
|                |                      |         | Completed | NCT00497497 | CRC            |
|                |                      |         | Completed | NCT00517049 | NHL            |
|                |                      |         | Completed | NCT00851136 | Metastatic CRC |
| LBY135         | TRAILR2/DR5          | IgG1    | Phase 2   | NCT01529307 | Advanced solid tumors |
|                |                      |         | Terminated| NCT01220999 | Advanced solid tumors |
|                |                      |         | Ongoing   | NCT01033240 | Liver cancer   |
|                |                      |         | Terminated| NCT00320827 | Malignancies, lymphoma |
| TAS266         | TRAILR2/DR5          | Tetrameric nanobody | Phase 1 | Terminated | NCT01529307 | Advanced solid tumors |

Abbreviations: CLL, chronic lymphocytic leukemia; CRC, colorectal cancer; HNC, head and neck cancer; NHL, non-Hodgkin lymphoma; NSCLC, non-small cell lung cancer; OC, ovarian cancer

Figure 3  TNFRSF receptor activation by oligomerized and FcγR-bound dimeric antibodies. The binding of two TNFRSF molecules by a bivalent antibody may lead, to some extent, to the recruitment of TNFRSF-associated proteins but with lower efficiency than in the case of stimulation by trimeric ligand. There is, however, no transactivation of TNFRSF receptor-associated signaling complexes. Optimal recruitment of adapter proteins as well as transactivation of receptor-bound effector molecules, thus full receptor activation, only occurs after secondary crosslinking of antibody–TNFRSF receptor complexes by protein A or G or secondary antibodies (a) or can be promoted by the self-affinity of the TNFRSF receptors when there is assistance by the spatial and mobility constraints given by binding to plasma membrane localized FcγRs (b)
decay receptors, TRAILR1 and TRAILR2 are typical representatives of the death receptor type of TNFRSF receptors. Early on, it has been observed that TRAIL triggers apoptosis in a variety of transformed cell lines but not or only rarely in non-transformed cell types. Accordingly, there were considerable efforts of a variety of research groups and companies to develop TRAIL death receptor-targeting therapeutics for tumor treatment. Indeed, recombinant soluble TRAIL (Dulanermin) and several TRAIL death receptor-specific antibodies have been subjected to clinical trials (Table 4). As monotherapy but also in combination with other anticancer drugs, all these TRAIL death receptor-targeting therapeutics have found to be well tolerated to date. Unfortunately, however, there was also no or quite limited clinical efficacy. From the beginning a variety of in vitro studies demonstrated that oligomerization potentiates the activity of soluble TRAIL (e.g., Schneider et al. and Wiley et al.) and TRAILR1/2 targeting antibodies (see above). Thus, the TRAIL death receptor-targeting reagents tested so far in the clinic obviously failed to unleash the full apoptotic activity of the two TRAIL death receptors and the poor therapeutic activity, but also the excellent tolerability, is therefore perhaps no real surprise. It is noteworthy that in accordance with the already discussed fact that poorly active soluble TNFSF ligand trimers can co-operate with barely active TNFRSF receptor-specific antibodies to trigger maximal receptor activation, it has been recently shown in vitro and in vivo that co-treatment with soluble TRAIL and the TRAILR2-specific antibody AMG655 (Conatumumab) results in enhanced apoptosis induction and improved antitumor responses. Soluble TRAIL and the murine TRAILR2-specific antibody MD5-1 also synergistically induce cell death in vitro in various murine cell lines. More importantly, the combined treatment with these reagents showed superior antitumor activity and good tolerability in vivo. This suggests that it is possible to target at least TRAILR2 with highly active agonists without paying with detrimental off-target effects.

TNFRSF Receptor Activation by Fcy Receptor-Bound Antibodies

TNFRSF receptor-specific bivalent antibodies not only resemble soluble TNFSF ligands with respect to the agonistic activity-potentiating effect of oligomerization but also mirror the differential ability of soluble and membrane-bound TNFSF ligands to activate certain types of TNFRSF receptors. Similar to soluble TNFSF ligand fusion proteins that functionally mimic membrane TNFSF ligands upon anchoring to cell surface-exposed molecules (Figure 3b), antigen-bound antibodies naturally anchor to certain cell types in an antigen-independent manner by interaction with Fc receptors recognizing the constant parts of antibodies. For the clinically most important IgG isotypes, there are five human and four murine Fc receptors, the so-called Fcy receptors (FcγR; Table 5) that are expressed to a varying extent on B cells and myeloid cell types. After binding of antigen–antibody complexes the activatory Fcy receptors (human: FcγRIIa, FcγRIIa, FcγRIIC, FcγRIIIA, FcγRIIB; murine: FcγRI, FcγRII, FcγRIII, FcγRIV) trigger immune effector functions, such as cytokine release, phagocytosis, antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). The activity of these activatory Fcy receptors is antagonized by the inhibitory FcγRIIB. There is now broad in vitro and in vivo evidence that Fcy receptor-bound antibodies display strongly enhanced agonistic activity. Crystallographic studies showed that a single IgG molecule interacts with a single FcγR molecule arguing against activation of TNFRSF receptors by sole FcγR-mediated cross-linking of receptor–antibody complexes as discussed above for protein A and secondary antibodies. Instead, it is tempting to speculate that in analogy to membrane-bound TNFSF ligands and cell surface anchored fusion proteins of soluble TNFSF ligands, the plasma membrane-associated spatial and mobility constraints of FcγR-bound antibodies assist TNFRSF receptor self-affinity driven clustering of receptor–antibody complexes (Figure 3b).

The potential relevance of FcγR binding for TNFRSF antibody activity in vivo became already indirectly obvious in the early studies with antibody class switch variants of the CD95 targeting APO-1 antibody. While it turned out that the IgG2b isoform of APO-1 is inactive in vitro, it nevertheless displayed significant antitumor activity in vivo. Although, it was not clarified in an early report to which extent antibody-dependent-effector functions, such as ADCC and CDC, and FcyR binding-dependent agonistic activity of APO-1 IgG2b contributed to the antitumoral effect, in vitro studies performed with the hamster IgG2 anti-mouse CD95 mAb Jo2 revealed later strong FcγR binding-dependent agonistic activity. Most importantly, however, in vivo studies with Jo2 and various mice strains with defective expression of one or more FcγRs revealed a crucial role of the inhibitory FcγRII receptor in Jo2-induced hepatotoxicity, the deadly hallmark of systemic CD95 activation. This straightforwardly showed for the first time that the FcγR binding-dependent agonistic activity of a TNFRSF receptor-specific IgG antibody, and thus receptor activation, is decisive for the observed in vivo effects.

Some important factors that determine the FcγR binding-dependent agonistic activity of TNFRSF receptor-specific antibodies have been revealed in recent years in preclinical studies by investigating the mode of action of CD40- and TRAILR2-specific antibodies by help of FcγR-deficient mice and FcγR discriminating antibody panels. In a vaccination model where the mouse CD40-reactive rat anti-CD40 IgG2a mAb 1C10 has been used as an adjuvant, Li and Ravetch observed abrogation of CD40-dependent T-cell expansion/activation and antitumor activity in mice without the common Fc receptor γ (FcγR) chain. As all three activating FcγRs in mice require the common FcγR chain for expression and signaling, this observation pointed to a crucial role of the remaining inhibitory FcγRII for the adjuvant activity of 1C10 and ruled out a major role of ADCC. In line with the idea of a FcγRII-dependent mode of CD40 activation, it turned out furthermore that 1C10-derived Fab2 preparations and a deglycosylated form of 1C10, thus 1C10 variants that fail to interact with Fcγ receptors, elicit no adjuvant activity in this model, too. Similar findings were made with 3/23, another murine CD40-specific rat IgG2a. A chimeric murine IgG1 variant of 3/23, which significantly binds to FcγRII and the activating FcγRIII, showed in vitro and in vivo strong...
stimulatory effects on antigen-presenting cells (B cells, dendritic cells) that are indicative for CD40 activation. In contrast, a chimeric murine IgG2a variant of 3/23 displaying strong binding to the murine activating Fcγ receptors but only poor binding to FcγRII showed no or only marginal immune stimulatory activities. Analogous results were also revealed in studies with the murine TRAILR2/DR5-specific hamster IgG2 antibody MD5-1 and the human TRAILR2/DR5-specific human IgG1 Drozitumab. Again, the activating FcγRs were found to be dispensable for agonistic antibody activity in vivo. A murine IgG1 variant of Drozitumab, which does not interact with FcγRIV, retained antitumoral activity in FcγRI/FcγRII double deficient mice. Similarly, the well-documented mouse strain-specific hepatotoxicity and tumoricidal activity of MD5-1 was completely abrogated in FcγRII mice. Moreover, Fc domain mutants of MD5-1 and Drozitumab devoid of Fcγ binding lost in vivo activity and a variant of MD5-1 with enhanced binding to human FcγRIIB showed improved activity in FcγRII KO mice with a human FcγRIIB transgene.

It is worth note that upon immobilization on plastic the aforementioned murine 3/23 chimeras were highly effective with respect to triggering CD40 activation irrespective of their FcγR preferences. In vitro studies with cells expressing a cytoplasmic deletion mutant of FcγRII indicated furthermore that triggering of intracellular signaling pathways is dispensable for FcγRII to unleash the agonistic activity of 3/23. Last but not least, it has been shown that all the activating FcγRs also promote CD40 activation by anti-CD40 IgGs and TRAILR2 activation by Drozitumab in vitro and a similar FcγR type-independent enhanced activity of FcγR-bound IgGs have also been reported for Fn14-specific antibodies.

### Table 5 Fcγ receptors

| Human Fcγ receptors | Murine Fcγ receptors |
|---------------------|----------------------|
| FcγRI CD64  | FcγRIIA CD32A  | FcγRIIB CD32B  | FcγRIIC CD32C  | FcγRIIIA CD16A  | FcγRIIB CD16B  | FcγRI CD16  | FcγRIIB CD16  | FcγRIIA CD16 | FcγRIIB CD16 |
| Yes  | No  | No  | No  | Yes  | No  | Yes  | No  | Yes  | Yes  |
| Effect  | activating  | activating  | inhibitory  | activating  | Activating  | Activating  | Inhibitory  | activating  | activating  |
| Main Expression  | DCs  | Monos  | Myeloid  | cell types  | Macros  | DCs  | Monos  | Macros  | Neutros  | Neutros  | Neutros  | DCs  | Myeloid  | cell types  | NK cells  | NK cells  | Monos  | Macros  | Neutros  |
| K0  | 8.8 nM  | 0.8-1 nM  | 0.29 μM  | 1.7 μM  | 6 μM  | 8.3 μM  | 9-11 μM  | 0.9 μM  | 0.5 μM  | 0.44 μM  | 5 μM  | 4.5 μM  | 0.1 μM  |
| K1  | 205 μM  | 2.2 μM  | 10 μM  | 1.2 μM  | 50 μM  | 33 μM  | 14 μM  | 55 μM  | n.m.  | n.m.  | 9 μM  | 1.1 μM  |
| K0  | 3.3 nM  | 1.1 μM  | 11 μM  | 1.1 μM  | 5.9 μM  | 0.12 μM  | 0.1 μM  | n.m.  | n.m.  | n.m.  | n.m.  | n.m.  |
| K0  | 26.2 nM  | 5.9 μM  | 4.8 μM  | 5 μM  | 5 μM  | 5 μM  | 4 μM  | n.m.  | n.m.  | n.m.  | n.m.  | n.m.  |

K0 < 10 nM, high affinity  
K0 > 10 nM and < 1 μM, medium affinity  
K0 > 1 μM, low affinity

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58See Lu et al. 78  
59See Luo et al. 160  
60See Bruhns et al. 161  
61See Nimmesgern et al. 162  
62See Vafa et al. 163  
63See White et al. 164  
64See White et al. 165  
65H131 allele of FcγRIIA  
66R131 allele of FcγRIIA  
67F158 allele of FcγRIIB  
68V158 allele of FcγRIIB  
69Human FcγRIIB variant NA1 (R36 N65 D82 V106)  
70Human FcγRIIB variant NA2 (S36 S65 N82 I106)
At the first glance, in sum these data suggest that the sole binding of dimeric antibodies to cell surface-expressed molecules or a plastic surface is sufficient to enable these molecules to activate TNFRSF receptors. However, this simple view is challenged by the observation that inhibitors of the actin cytoskeleton strongly inhibit the receptor-stimulating activity of CD95- and DR5-specific IgG antibodies without affecting their binding to FcγRs.

Against the background that binding to all FcγR types is sufficient to confer strong agonistic activity to TNFRSF receptor-specific antibodies in vitro, it is tempting to speculate that the observed dominant role of the inhibitory FcγRII in vivo reflects its better bioavailability compared with the activating FcγRs. In further accordance with the idea that the available number of Fcγ receptors is important for the in vivo activity of dimeric anti-TNFFRSF receptor antibodies, Li and Ravetch reported that the agonistic in vivo activities of the CD40-specific 1C10 and the TRAILR2-specific mAb MD5-1 are abrogated not only in FcγRII KO mice but also in heterozygous FcγRII–/– animals.

Taked together, FcγR-bound bivalent antibodies display high, membrane-bound TNFSF ligand mimicking TNFRSF receptor-stimulating activity and resemble in this regard extracellular matrix-bound soluble TNFSF ligands and soluble TNFSF ligand fusion proteins that have been anchored to a cell surface-expressed molecular target. Of course, this does not mean that ‘conventional’ Fc effector activities of antibodies, such as ADCC or CDC, are unimportant for the in vivo effects of TNFRSF receptor-specific antibodies. Indeed, the antimortal activity of IgGs targeting the costimulatory TNFSF receptors GITR and OX40 have been found to be dominated by ADCC of tumor-associated regulatory T cells.

**Conclusion and Perspective**

The knowledge accumulated in recent years on the relevance of valency, oligomerization and FcγR binding for the agonistic activity of TNFRSF receptor-targeted antibodies will certainly improve the rational design of antibody-derived TNFRSF receptor agonists but will also help to avoid pitfalls. The agonism-generating effects of oligomerization and FcγR binding are also of obvious relevance for the development of antagonistic ligand binding-blocking TNFRSF antibodies. Corresponding efforts have not only to avoid the use of antibody variants that bind FcγRs but must also ensure lack of immunogenicity to prevent the development of cross-linking secondary antibodies.

The recognition of the overwhelming importance of FcγRII/FcγRIIB binding for the agonistic activity of most TNFRSF receptor-specific IgGs may revitalize/enhance efforts to target the TRAIL death receptors in cancer therapy with antibody variants with FcγRII-binding properties superior to the antibodies used so far. In cases where FcγRIIB anchoring has its limitations, for example, due to poor bioavailability of FcγRIIB expressing cells, artificial oligomerization of TNFRSF receptor-specific antibodies or antibodies fragments may deliver an alternative solution to overcome the poor agonistic activity of conventional IgGs. Indeed, high, secondary oligomerization-independent activity has been described for trimeric, tetrameric and pentameric TRAILR2/DR5-specific nanobody/scFv variants. A first clinical trial with the tetravalent nanobody TAS266 revealed reversible hepatotoxicity. Thus, multivalent highly active TRAILR2-targeting antibody constructs may offer the promise of increased antitumoral activity but there is also a need to reconsider the possible side effects of systemic TRAILR2 activation when potent agonists are used in vivo.

The relevance of oligomerization and FcγRIIB anchoring for the agonistic activity of bivalent TNFRSF receptor-specific antibodies has been clearly recognized yet and corresponds very well with current concepts of TNFRSF receptor activation by secondary interaction of TNFSF ligand3–5–TNFRSF receptorR complexes. Oligomerization and FcγRIIB anchoring of bivalent antibodies, however, are presumably not the only factors that determine agonistic activity of TNFRSF-specific IgGs. There are at least two basal observations that cannot be straightforwardly integrated in a TNFRSF receptor activation model where oligomerized and cell surface-anchored IgGs promote the clustering of TNFSF ligand3–5–TNFRSF receptorR complexes. First, only just, an unexpected, clinically potentially relevant, FcγR binding-independent agonistic activity has been observed for CD40-targeting human IgG2 isoform B antibodies. Here, future studies must show whether this type of bivalent antibody indeed activates TNFRSF receptor-associated pathways without TNFRSF receptor clustering or have to clarify how this antibody type triggers TNFRSF receptor clustering without an obvious capacity to auto-aggregate and without evidence for antigen-independent cell surface binding. Second, it is currently not understood why the agonistic activity of FcγR-bound CD95- and TRAILR2/DR5-specific IgG antibodies is abrogated by pretreatment of the FcγR-expressing cells with actin inhibitors although this do not interfere with antibody binding.

**Conflict of Interest**

The author declares no conflict of interest.

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