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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.
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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 is the molecular basis of the different responsiveness of TNFRSF receptors toward binding of soluble TNFSF ligands?
- How one can generate antibody-based TNFRSF receptor agonists with oligomerization- and FcγR binding-independent activity?
- 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 (LT$\alpha\beta_2$)[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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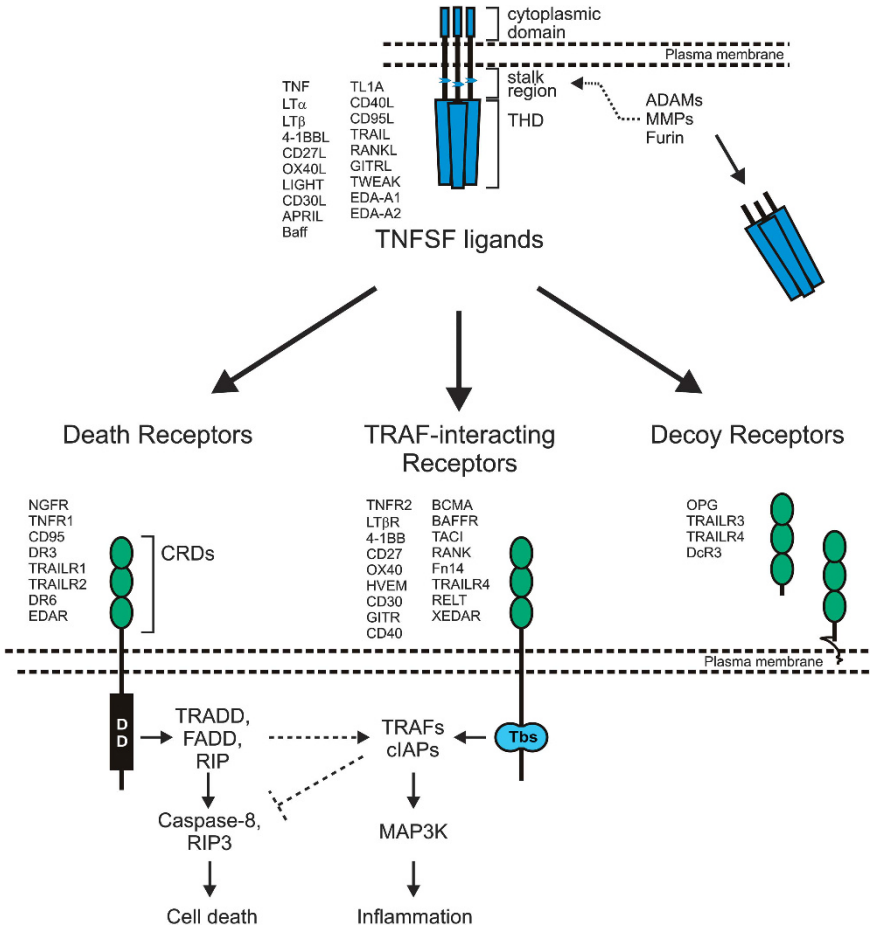

Figure 1 Ligands of the TNF superfamily (TNFSF) stimulate receptors of the TNF receptor superfamily (TNFRSF). The TNFSF comprises 19 human ligands, which are defined by a conserved C-terminal trimerization domain, designated as TNF homology domain (THD), and include TNF, CD40L, CD95L and TWEAK. LTα is a secreted ligand while the other TNFSF ligands are single spanning transmembrane proteins. In many cases, however, soluble ligand molecules can be released from the membrane-bound proteins by proteolytic cleavage in the stalk region by proteases of the furin, matrix metalloproteinase (MMP) and a disintegrin and metalloproteinase[89] family. TNFSF ligands exert their activity by stimulation of TNFRSF receptors. The latter are characterized by having one or more cysteine-rich domains (CRDs) in their extracellular parts and can be classified into three groups according to functional and structural similarities: (i) death receptors that have a cytoplasmic protein–protein interaction domain called death domain that enables some death receptors to trigger cell death pathways, (ii) TRAF-interacting receptors that contain one to three binding motifs for adapter proteins of the TNF receptor-associated factor (TRAF) family that link these receptors to proinflammatory signaling pathways, and (iii) decoy receptors without own signaling capabilities that control the activity of other TNFRSF receptors. With regard to function the classification of the signaling competent TNFRSF receptors into cell death-inducing death receptors and proinflammatory TRAF-interacting receptors is an oversimplification. Death receptors are also able to trigger proinflammatory pathways and TRAF-interacting receptors via versa can boast apoptotic responses by blocking TRAF-dependent survival activities

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$_3$–TNFRSF receptor complex then recruits in two further steps two additional monomeric TNFRSF receptor molecules to form an active TNFSF ligand$_3$–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

Table 1 Crystal structures of ligands and receptors of the TNF family

Structure	PDB ID	Resolution (Ä)	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 LTαβ_2-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	2HEY	2	108
Human OX40L-humanOX40	2HEV	2.41	108
Human 4-1BBL	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
	1D4V	2.2	114
Murine RANKL	1JTZ	2.6	115
	1S55	1.9	not recorded in Pubmed
	1IQA	2.2	116
Murine RANK	3ME4	2.01	117
Human RANKL-OPG	3URF	2.7	118
Murine RANKL-RANK	3QBQ	2.5	119
	4GIQ	2.7	7
	3ME2	2.8	117
Murine RANKL-OPG	4E4D	2.7	7
Murine GITRL	2Q8O	1.75	4
	3FC0	1.76	120
	3B91, 2QDN	2.49, 2.09	3
Human GITRL	2R32, 2Q1M	1.95, 2.3	121
Human TL1A	2QE3	2.5	122
	2RE9, 2O0O	2.1, 3	123
Human DcR3	3MHD	2.9	124
Human TL1A-DcR3	3MI8, 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	1U5Z	2.4	126
	1U5Y	2.3	126
	1U5X	1.8	126
Human Baff	1JH5	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-BaffR	1OQE	2.5	133
	1OTZ, 1P0T	3.3	134
Human Baff-BCMA	1OQD	2.6	133
Human APRIL-TACI	1XU1	1.9	131
Human APRIL-BCMA	1XU2	2.35	131
Human Fn14	2KMZ	Solution NMR	132
	2RPJ	Solution NMR	135
Xenopus Fn14	2KN0	Solution NMR	132
Human EDA-A1	1RJ7	2.3	136
Human EDA-A2	1RJ8	2.23	136
Human DR6	3QO4	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	3IJ2	3.75	140
	1SG1	2.4	141

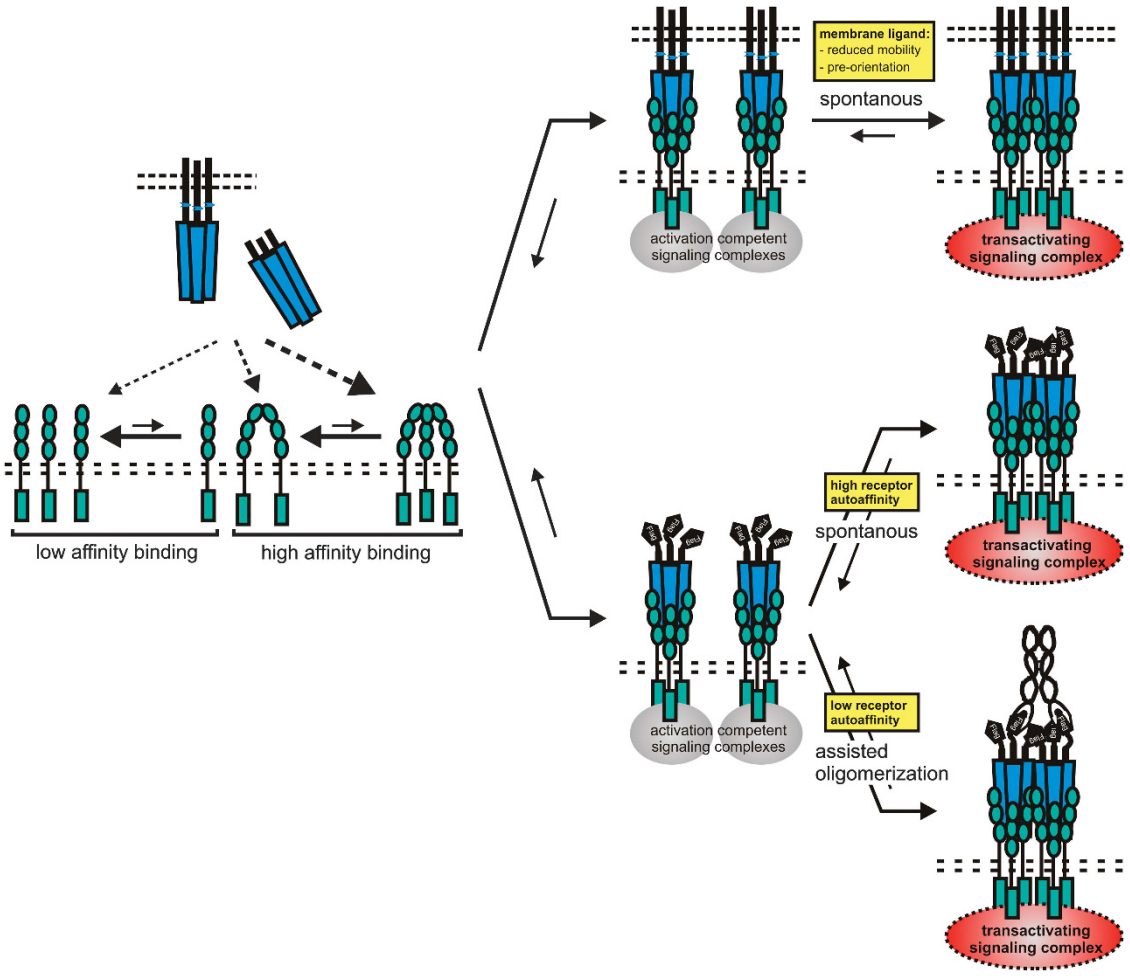

Figure 2 PLAD-assisted oligomerization model of TNFRSF receptor activation. This model is based on the fundamental observation that at least some TNFRSF receptors pre-assembles in the absence of ligand. The self-affinity of TNFRSF receptors would not only allow to explain TNFSF ligand binding by formation of high affinity dimeric or trimeric TNFRSF complexes but may also drive secondary interaction of TNFSF ligand$_3$–TNFRSF receptor$_3$ complexes. The initially formed TNFSF ligand$_3$–TNFRSF receptor$_3$ 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$_3$–TNFRSF receptor$_3$ 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

within several TNFRSF receptors that mediates self-assembly in the absence of ligand molecules.[9,11–13] The interaction of two (or three) receptor molecules by this so-called 'pre-ligand assembly domain' (PLAD) may create single high affinity binding sites for TNFSF ligand trimers. This not only explains the single binding site interaction typically found for TNFSF ligands and cell bound TNFRSF receptors but also delivers a rationale for the dominant-negative activity of ligand binding-defective CD95/TACI mutants. If such mutants still contain a functional PLAD, then this results in the trapping of wild-type receptor molecules in complexes with mutant receptor molecules. The latter do not contribute to ligand binding, thus in this case dimerization of receptor molecules does not result in a relevant increase in apparent affinity. It is noteworthy that the affinity of the PLAD–PLAD interaction is rather low and almost in the mM range.[14] This corresponds to the observation that soluble TNFRSF receptor ectodomains are typically very poor TNFSF ligand agonists unless they are fused with multimerizing scaffolds. In view of the weak PLAD-PLAD affinity an unclear aspect of the PLAD-based TNFRSF receptor activation model concerns the equilibrium between monomeric and PLAD-assembled TNFRSF receptors. At one extreme, the PLAD-PLAD affinity, despite its weakness, is possibly sufficient to drive the huge majority of receptors in the PLAD-assembled state due to the spatial pre-orientation and immobilization of the receptor molecules in the plasma membrane (Figure 2b). However, at the other extreme, the equilibrium point favors monomeric TNFRSF receptors and suggests that there are only a few receptors in the PLAD-assembled state at any given moment (Figure 2b). In this second scenario, the binding of a TNFSF ligand trimer to the rare PLAD-assembled receptor species would result in the stabilization of the few assembled receptors and their removal from the equilibrium with the monomeric receptor species. According to the principle of LeChatelier, the pool of ligand-free PLAD-assembled TNFRSF receptors is then recovered at the expense of the pool of the monomeric receptor species. Thus, with time almost the complete pool of TNFRSF receptor

Table 2 Effect of anti-Flag oligomerization on the receptor stimulating activities of soluble Flag-tagged TNFSF ligand trimers

TNFSF ligand	Cellular response	EC50 $_{w/o\ crosslink}$ / EC50 $_{with\ crosslink}$	Ref.
CD95L	Cell death	100 - >> 1000	72,142
TRAIL	Cell death	70 - >> 1000	72,73,142,143,144
TWEAK	Cell death	1	72,145
	p100 processing	1	22,72,146
	IL8	> 100	22,72,146
TNF to TNFR1	Cell death		72
TNF to TNFR2	Proliferation	Differ in max. response	72
EDA1[a]	Cell death[b]	100 - 1000	147
CD40L	Proliferation	>> 1000	18
	IL8	5-25	148
APRIL to TACI	Proliferation, MHC II induction	~ 50 - > 100	17,18
Baff	Proliferation	10-20	18
Baff to BCMA[c]	Cell death	> 100	17
Baff to BCMA	NFκB reporter	> 1000	17
OX40L	IL8	~ 100	149
41BBL	IL8	>> 100	148
CD27L	IL8	>> 100	148
GITRL	IL8	~ 5	148

[a]Soluble trimeric variant without oligomerizing collagen domain
[b]Transfectants expressing an artificial EDAR-CD95 chimeric receptor
[c]Transfectants expressing an artificial BCMA-CD95 chimeric receptor

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 ligand$_3$–TNFRSF receptor$_3$ 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 TACI and Baff receptor-3 (BR3) but only activates the latter.[17,18] 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 ligand$_3$–TNFRSF receptor$_3$ 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 ligand$_3$–TNFRSF receptor$_3$ complexes for activation. The need for clustering of TNFSF ligand$_3$–TNFRSF receptor$_3$ 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 Wajant[21]). 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 homotrimeric molecules 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 conformation with E3 activity and the capacity to 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 (TWEAK$_3$-Fn14$_3$-TRAF2$_3$-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 cytosol[32,33] where they are involved in triggering the destruction of the alternative NFκB inducing kinase NIK. In the case of CD95-

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

TNFSF fusion protein	Number of TNF trimers	Examples	$\frac{EC50_{trimer}}{EC50_{fusion\ protein}}$	Ref.
Fc-TNFSF	2	CD95L	1000	150
		OX40L	~ 10	149
		TWEAK[a]	> 100	22
ACRP-TNFSF	2	CD95L	100	150
		CD40L	> 100	150
Fc-scTNFSF	2	TRAIL	> 100	151
EDH2-scTNFSF	2	TRAIL	> 10 - 100	152
TNC-scTNFSF	3	TNFR2-specific TNF	inactive versus highly active	153
Fc-TNC-TNFSF	2	4-1BBL	~ 100	148
SP-D-TNFSF	4	CD40L	Improved max. responses	154,155
		Baff	highly active[b]	156
		4-1BBL	highly active[b]	156
		OX40L	highly active[b]	157
Fc-ILZ-TNFSF	2	OX40L	highly active[b]	158

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
[a]The enhancing effect observed in this study depends on the TWEAK-induced pathway considered. Fc-TWEAK showed a 100-fold lower EC50 for classical NFκB signaling compared with Flag-TWEAK while both molecules were equally effective in triggering p100 processing
[b]Soluble TNFSF ligand trimers have not been analyzed

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 $CD95L_3$–$CD95_3$ 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 $ligand_3$–TNFRSF $receptor_3$ complexes, which might already trigger certain signaling pathways. In a second step, there is then oligomerization of TNFSF $ligand_3$–TNFRSF $receptor_3$ complexes that eventually enables activation of signaling pathways requiring transactivation/oligomerization of TNFSF $ligand_3$–TNFRSF $receptor_3$ 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 supramolecular 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 $CD95L_3$–$CD95_3$ 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.[42,43] Similarly, soluble CD95L gain high apoptotic activity after fibronectin binding and APRIL stimulates Baff-R when trapped by the extracelluar 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]

Ligand binding and self-assembly occur via different parts of the ectodomain of TNFRSF receptors.[9,11] TNFRSF receptors have therefore the ability to interact with each other also when complexed by their ligand suggesting a model of TNFRSF receptor activation in which PLAD–PLAD interactions not only facilitate the binding of TNFSF ligands to TNFRSF receptors to form signaling competent TNFSF $ligands_3$–TNFRSF $receptors_3$ complexes but also promote secondarily their clustering into supramolecular aggregates where transactivation of TNFRSF $receptor_3$-associated signaling complexes become possible (Figure 2b).

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 $ligand_3$–TNFRSF $receptor_3$ complexes. However, it cannot be ruled out that this TNFRSF receptor type uses still unknown

mechanisms/factors enabling these receptors to promote oligomerization of TNFRSF-associated adapter proteins without oligomerization of TNFSF ligand$_3$–TNFRSF receptor$_3$ 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[47–49] 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.[50] Likewise, it was found that cross-linking converts the antagonistic TNFR1-specific IgG2a antibody H398 into a potent TNFR1 agonist.[51] 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.[52] 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*.[53] Cross-linking with protein A or secondary antibodies, however, restored the high agonistic activity of these APO-1 variants.[53] 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 Fab$_2$ fragments derived of this antibody, has high, aggregation-independent agonistic activity.[54–56] 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,[57,58] CD27,[59] TRAILR1/DR4,[60] TRAILR2/DR5[61–65] and Fn14.[24,66–68] 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.[58] 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.[69] 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$_2$–antibody complexes is required to form active [receptor$_2$–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.[15] 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.[58,70] 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$_2$–

Table 4 TNFRSF receptor antibodies in clinical trials

Antibody	Target	Isotype	Status		ID	Condition
Brentuximab-Vedotin SGN-35	CD30	Drug conjugate, chimerized IgG1	Approved, >70 studies		—	Lymphoma
XmAb2513	CD30	IgG1 Enhanced FcγR binding	Phase 1	Completed	NCT00606645	Hodgkin lymphoma Anaplastic large cell lymphoma
MDX-1401	CD30	IgG1	Phase 1	Completed	NCT00634452	Hodgkin lymphoma
HeFi-1	CD30	Murine IgG1	Phase 1	Completed	NCT00048880	Neoplasms
		Agonist	Phase 1	Completed	NCT00003741	Lymphoma
PF-05082566	4-1BB	IgG2	Phase 1	Recruiting	NCT02179918	Advanced solid tumors
		Agonist	Phase 1	Recruiting	NCT01307267	NHL
Urelumab BMS-663513	4-1BB	IgG4 Agonist	Phase 1	Recruiting	NCT01775631	B-cell malignancies
			Phase 1	Recruiting	NCT02252263	Multiple myeloma
			Phase 1/2	Recruiting	NCT02253992	Advanced solid tumors
			Phase 1	Recruiting	NCT01471210	Advanced B-cell NHL
			Phase 1	Recruiting	NCT02110082	Solid tumors, B-cell NHL
			Phase 1	Terminated	NCT00461110	CRC, HNC
			Phase 1	Terminated	NCT00351325	NSCLC
			Phase 1/2	Terminated	NCT00309023	Solid malignancies
			Phase 2	Completed	NCT00612664	Advanced cancer Melanoma
TRX518	GITR	IgG1 N297 Fc-disabled	Phase 1	Recruiting	NCT01239134	Stage III/IV melanoma Solid tumors
MK-4166	GITR		Phase 1	Recruiting	NCT02132754	Solid tumors
Varlilumab CDX-1127	CD27	IgG1	Phase 1	Recruiting	NCT01460134	B-cell malignancies Solid tumors
			Phase 1	Recruiting	NCT02284971	Prostate cancer
			Phase 1/2	Recruiting	NCT02335918	NSCLC, CRC, HNC, OC, Melanoma
MEDI6469	OX40	Murine IgG1	Phase 1	Unknown	NCT01644968	Advanced cancer
			Phase 1/2	Recruiting	NCT01862900	Metastatic breast, lung and liver cancer
			Phase 1	Recruiting	NCT02274155	HNC
			Phase 1/2	Recruiting	NCT01303705	Prostate cancer
			Phase 1/2	Recruiting	NCT02205333	Advanced solid tumors
MEDI0562	OX40	IgG1 humanized Agonist	Phase 1	Recruiting	NCT02318394	Solid tumors
CP-870,893	CD40	IgG2	Phase 1	Completed	NCT01103635	Recurrent/IV melanoma
		Agonist	Phase 1	Completed	NCT00607048	Neoplasms
			Phase 1	Completed	NCT02225002	Advanced solid tumors
			Phase 1	Active	NCT01008527	Melanoma
			Phase 0	Completed	NCT02157831	Solid tumors
			Phase 1	Completed	NCT01456585	Adenocarcinoma
			Phase 1	Completed	NCT00711191	Pancreatic neoplasm
PG102 FFP104	CD40	IgG4 Antagonist		Terminated (poor recruitment)	NCT00787137	Psoriatic arthritis
Lucatumumab HCD122	CD40	IgG1	Phase 2	Completed	NCT00231166	Multiple myeloma
		Antagonist	Phase 1	Terminated	NCT00108108	CLL
Chi Lob 7/4	CD40	IgG1 chimeric Agonist	Phase 1	Completed	NCT01561911	Cancer, lymphoma
ASKP1240	CD40	IgG4	Phase 1	Completed	NCT01565681	Healthy volunteers
		Antagonist	Phase 2	Completed	NCT01585233	Psoriasis
			Phase 2	Active	NCT01780844	Kidney transplantation
Enavatuzumab PDL192	Fn14	IgG1 humanized	Phase 1	Completed	NCT00738764	Advanced solid tumors
Conatumumab AMG655	TRAILR2/DR5	IgG1	Phase 1b	Completed	NCT00791011	Lymphoma
		Agonist	Phase 1b/2	Completed	NCT00625651	CRC
			Phase 1b/2	Terminated	NCT00819169	Solid tumors
			Phase 1b/2	Completed	NCT00626704	Sarcoma
			Phase 1b/2	Completed	NCT00534027	NSCLC
			Phase 1b/2	Completed	NCT00630552	Pancreatic cancer
			Phase 2	Completed	NCT00813605	Metastatic CRC
			Phase 2	Ongoing	NCT01327612	Solid tumors, lymphoma
			Phase 1b/2	Completed	NCT00630786	CRC
Lexatumumab HGS-ETR2	TRAILR2/DR5	IgG1 Agonist	Phase 1	Completed	NCT00428272	Sarcoma neuroblastoma

Table 4 *(Continued)*

Antibody	Target	Isotype		Status	ID	Condition
Mapatumumab HGS-ETR1	TRAILR1/DR4	IgG1	Phase 2	Completed	NCT00092924	NSCLC
		Agonist	Phase 2	Completed	NCT00094848	NHL
Tigatuzumab	TRAILR2/DR5	IgG1 humanized	Phase 1	Completed	NCT01220999	CRC neoplasms
CS-1008		Agonist	Phase 2	Ongoing	NCT01307891	Breast cancer
			Phase 2	Terminated	NCT00969033	Metastatic CRC
			Phase 2	Completed	NCT00991796	NSCLC
			Phase 2	Completed	NCT00521404	Pancreatic cancer
			Phase 2	Completed	NCT00945191	OC
			Phase 1	Completed	NCT01124630	Metastatic CRC
			Phase 2	Ongoing	NCT01033240	Liver cancer
			Phase 1	Completed	NCT00320827	Malignancies, lymphoma
Drozitumab	TRAILR2/DR5	IgG1	Phase 2	Terminated	NCT00543712	Chondrosarcoma
PRO95780		Agonist	Phase 2	Completed	NCT00480831	NSCLC
			Phase 1	Completed	NCT00497497	CRC
			Phase 2	Completed	NCT00517049	NHL
			Phase 1	Completed	NCT00851136	Metastatic CRC
LBY135	TRAILR2/DR5	IgG1 chimeric	Sharma			Advanced solid tumors
		Agonist	*et al.*[159]			
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

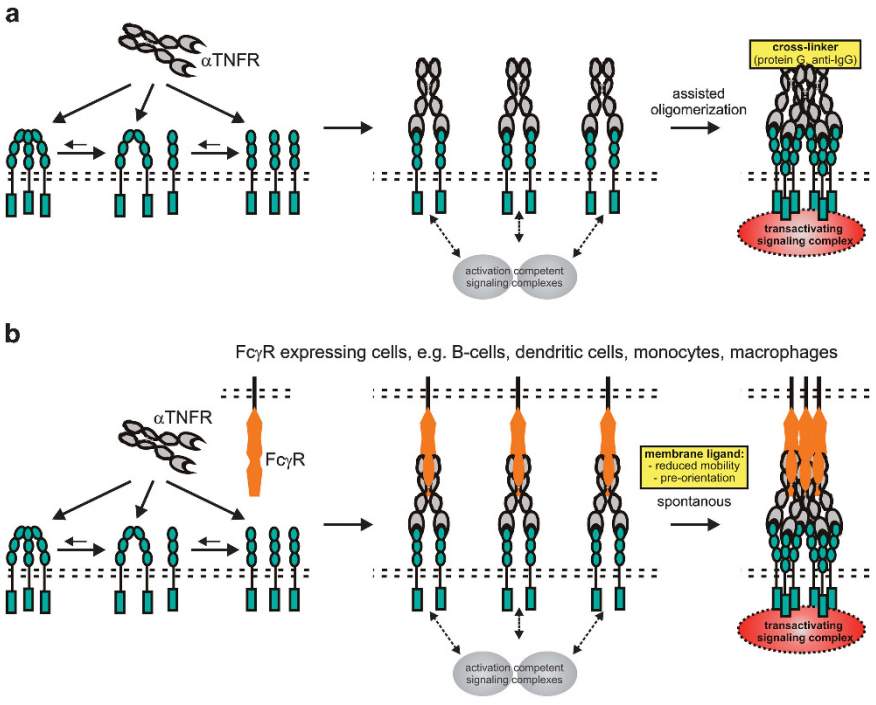

Figure 3 TNFRSF receptor activation by oligomerized and FcγR-bound dimeric antibodies. The binding of two TNFRSF molecules by a bivalent antibody may lead, to some extent, to the recruitment of TNFRSF-associated proteins but with lower efficiency than in the case of stimulation by trimeric ligand. There is, however, no transactivation of TNFRSF receptor$_3$-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$_2$ 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**)

antibody complexes and thus on receptor$_2$–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

decoy receptors, TRAILR1 and TRAILR2 are typical representatives of the death receptor type of TNFRSF receptors.[71] 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/are considerable efforts of a variety of research groups and companies to develop TRAIL death receptor-targeting therapeutics for tumor treatment.[71] 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.[71] 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.*[72] and Wiley *et al.*[73]) 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.[74,75] Soluble TRAIL and the murine TRAILR2-specific antibody MD5-1 also synergistically induce cell death *in vitro* in various murine cell lines.[74] More importantly, the combined treatment with these reagents showed superior antitumor activity and good tolerability *in vivo*.[74] 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 Fcγ 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 Fcγ receptors (FcγR; Table 5) that are expressed to a varying extent on B cells and myeloid cell types.[76,77] After binding of antigen–antibody complexes the activatory Fcγ receptors (human: FcγRI, FcγRIIA, FcγRIIC, FcγRIIIA, FcγRIIIB; murine: FcγRI, 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 Fcγ receptors is antagonized by the inhibitory FcγRIIB.[76,77] There is now broad *in vitro* and *in vivo* evidence that Fcγ receptor-bound antibodies display strongly enhanced agonistic activity. Crystallographic studies showed that a single IgG molecule interacts with a single FcγR molecule[78–82] arguing against activation of TNFRSF receptors by sole FcγR-mediated cross-linking of receptor$_2$–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$_2$–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*.[53] Although, it was not clarified in an early report to which extent antibody-dependent effector functions, such as ADCC and CDC, and FcγR 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.[83] 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.[84,85] 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[86] observed abrogation of CD40-dependent T-cell expansion/activation and antitumor activity in mice without the common Fc receptor γ (FcRγ) chain. As all three activating FcγRs in mice require the common FcRγ 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 Fab$_2$ 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.[86] 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

Table 5 Fcγ receptors

	Human Fcγ receptors						Murine Fcγ receptors			
	FcγRI CD64	**FcγRIIA CD32A**	**FcγRIIB CD32B**	**FcγRIIC CD32C**	**FcγRIIIA CD16A**	**FcγRIIIB CD16B**	**FcγRI**	**FcγRIIB**	**FcγRIII**	**FcγRIV**
FcRγ use	yes	No	No	no	Yes	No	Yes	No	yes	yes
Effect	activating	activating	inhibitory	activating	Activating	activating	activating	Inhibitory	activating	activating
Main Expression	DCs Monos Macros	Myeloid cell types	B-cells Basophils DCs	Monos Macros Neutros NK cells	Monos Macros NK cells	Neutros	DCs	B-cells Myeloid cell types	NK cells Myeloid cell types	Monos Macros Neutros
K_D hIgG1	8.8 nM[a] 0.9-1 nM[b] 15 nM[c] 20 nM[e]	0.19 μM[c,h] 0.29 μM[c,i] 1.7 μM[e] 6 μM[b]	8.3 μM[c]		9-11 μM[b] 0.9 μM[c,j] 0.5 μM[c,k] 0.44 μM[e]	5 μM[c,l] 4.5 μM[c,m]				0.1 μM[d]
K_D hIgG2	205 μM[e]	2.2 μM[c,h] 10 μM[c,i] 1.2 μM[e]	50 μM[c]		33 μM[c,j] 14 μM[c,k] 55 μM[e]	n.m[c]				
K_D hIgG3	3,3 nM[a] 16 nM[c]	1.1 μM[c,h] 1.1 μM[c,i]	5.9 μM[c]		0.12 μM[c,j] 0.1 μM[c,k]	9 μM[c,l] 1.1 μM[c,m]				
K_D hIgG4	26.2 nM[a] 29 nM[c]	5.9 μM[c,h] 4.8 μM[c,i]	5 μM[c]		5 μM[c,j] 4μM[c,k]	n.m[c]				
K_D mIgG1							n.m[f]	0.33μM[d] 0.17 μM[f] 0.58 μM[g]	3.2 μM[d] 0.32 μM[f]	n.m[d,f]
K_D mIgG2a							20 nM[f]	2.4 μM[d]	1.5 μM[d]	34.5 nM[d]
							45 nM[g]	1.5 μM[g]	0.14 μM[f] 0.38 μM[g]	12 nM[f] 32 nM[g]
K_D mIgG2b								0.45 μM[d]	1.6 μM[d]	59 nM[d]
K_D mIgG3								n.m[d]	n.m[d]	n.m[d]

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

[a]See Lu *et al.*[78]
[b]See Luo *et al.*[160]
[c]See Bruhns *et al.*[161]
[d]See Nimmerjahn *et al.*[162]
[e]See Vafa *et al.*[163]
[f]See White *et al.*[87]
[g]see White *et al.*[164]
[h]H131 allele of FcγRIIA
[i]R131 allele of FcγRIIA
[j]F158 allele of FcγRIIIA
[k]V158 allele of FcγRIIIA
[l]Human FcγRIIIB variant NA1 (R36 N65 D82 V106)
[m]Human FcγRIIIB variant NA2 (S36 S65 N82 I106)

stimulatory effects on antigen-presenting cells (B cells, dendritic cells) that are indicative for CD40 activation.[87] 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.[87] 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.[88,89] 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/FcγRIII double deficient mice.[89] Similarly, the well-documented mouse strain-specific hepatotoxicity and tumoricidial activity of MD5-1[90,91] was completely abrogated in FcγRII mice.[88] Moreover, Fc domain mutants of MD5-1 and Drozitumab devoid of FcγR 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.[88]

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.[87] *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.[87] 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.[24,68,87,89]

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.[83,89]

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-TNFRSF receptor antibodies, Li and Ravetch[92] 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.

Taken 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 antitumoral activity of IgGs targeting the costimulatory TNFRSF receptors GITR and OX40 have been found to be dominated by ADCC of tumor-associated regulatory T cells.[93,94]

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 FcγRIIB-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 oliogmerization 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.[95,96] A first clinical trial with the tetravalent nanobody TAS266 revealed reversible hepatoxicity.[97] 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 ligand$_3$–TNFRSF receptor$_3$ 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 ligand$_3$–TNFRSF receptor$_3$ 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.[98] 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.[83,89]

Conflict of Interest

The author declares no conflict of interest.

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