

Binding Studies of TNF Receptor Superfamily (TNFRSF) Receptors on Intact Cells*

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Ligands of the tumor necrosis factor superfamily (TNFSF) interact with members of the TNF receptor superfamily (TNFRSF). TNFSF ligand-TNFRSF receptor interactions have been intensively evaluated by many groups. The affinities of TNFSF ligand-TNFRSF receptor interactions are highly dependent on the oligomerization state of the receptor, and cellular factors (e.g. actin cytoskeleton and lipid rafts) influence the assembly of ligand-receptor complexes, too. Binding studies on TNFSF ligand-TNFRSF receptor interactions were typically performed using cell-free assays with recombinant fusion proteins that contain varying numbers of TNFRSF ectodomains. It is therefore not surprising that affinities determined for an individual TNFSF ligand-TNFRSF interaction differ sometimes by several orders of magnitude and often do not reflect the ligand activity observed in cellular assays. To overcome the intrinsic limitations of cell-free binding studies and usage of recombinant receptor domains, we performed comprehensive binding studies with *Gaussia princeps* luciferase TNFSF ligand fusion proteins for cell-bound TNFRSF members on intact cells at 37 °C. The affinities of the TNFSF ligand *G. princeps* luciferase-fusion proteins ranged between 0.01 and 19 nM and offer the currently most comprehensive and best suited panel of affinities for *in silico* studies of ligand-receptor systems of the TNF family.

The ligands and receptors of the tumor necrosis factor (TNF) family constitute two complementary families of proteins that regulate a huge variety of immune processes but are also involved in the control of tissue homeostasis and differentiation of certain cell types (1, 2). Assignment to the TNF ligand family is based on the presence of a conserved C-terminal TNF homology domain (THD),² which mediates formation of homotrimeric molecules and binding to members of the TNF receptor family (1, 2). TNFSF ligands are typically type II or type III transmembrane proteins and thus contain in addition to the THD an N-terminal cytosolic domain and a single transmembrane domain that is separated from the THD by a stalk region

of variable length and structure (1, 2). There are also soluble variants of TNFSF ligands that result from proteolytic processing in the stalk region or from alternative splicing. Because of the presence of the THD, these soluble molecules also form trimers that retain the receptor-binding ability of the membrane-bound ligand.

The TNFRSF receptors are characterized by the presence of one or more copies of a conserved cysteine-rich domain in the N-terminal part of these molecules (1, 2). According to structural and functional similarities, three subcategories of TNFRSF receptors can be defined. The first subgroup comprises the death receptors that are characterized by a conserved C-terminally located protein-protein interaction domain, called the death domain (3). The death domain enables most death receptors to induce apoptosis but is also required to transmit non-apoptotic signals. Second, there are the TNF receptor-associated factor (TRAF)-interacting receptors that possess one or more motifs in their cytoplasmic domain allowing direct interaction with members of the TRAF adapter protein family (4). The third subgroup consists of soluble and membrane-anchored TNFRSF receptors that act as decoy receptors of the death and TRAF-interacting receptors by competition for ligand binding and/or by forming signaling-incompetent heteromeric receptor complexes (5, 6).

The broad physiological and pathophysiological relevance of the ligands and receptors of the TNF family is, for example, evident from the fact that various inherited diseases, including autoimmune lymphoproliferative syndrome (ALPS), common variable immunodeficiency, TNFR1-associated periodic fever syndrome, X-linked hyper-IgM syndrome, and X-linked hypohidrotic ectodermal dysplasia, can be caused by mutations in genes encoding ligand and receptors of the TNF family (CD40L (X-linked hyper-IgM syndrome), CD95L (ALPS), ectodysplasin-A1 (EDA-A1; XEDA), CD95 (ALPS), TACI (common variable immunodeficiency), TNFR1 (TNFR1-associated periodic fever syndrome)) (7). Moreover, chronic production of TNF and the deregulated activity of receptor activator of NF- κ B ligand have been identified as crucial factors in the development of autoimmune diseases and the progression of bone destructive processes (7). The specificity of TNFSF ligand-TNFRSF receptor interactions and the extracellular accessibility of these molecules make them highly attractive and straightforward targets for therapeutic interventions. Indeed, recombinant TNF is approved for the treatment of soft tissue sarcoma by isolated limb perfusion, and TNF-specific antibodies and a TNFR2 ectodomain Fc-fusion protein are an estab-

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² The abbreviations used are: THD, TNF homology domain; TNFSF, tumor necrosis factor superfamily; TNFRSF, TNF receptor superfamily; GpL, *G. princeps* luciferase; TRAF, TNF receptor-associated factor; ALPS, autoimmune lymphoproliferative syndrome; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; PLAD, pre-ligand assembly domain.

lished and broadly used option in the therapy of various immune diseases, including rheumatoid arthritis and Crohn disease (7). Furthermore, neutralizing antibodies specific for receptor activator of NF- κ B ligand and BAFF are approved for the treatment of osteoporosis and systemic lupus erythematosus, and several more molecules targeting ligands and receptors of the TNF family are under consideration in clinical trials.

The development and analysis of drugs modulating the activity of a certain TNFSF ligand-TNFRSF receptor pair as well as the understanding of the principles of TNF receptor activation highly benefit from knowledge of the affinities of these interactions. With few exceptions, however, experimental data on the receptor affinity of TNF ligands derived from analysis with intact cells are rare. Indeed, the majority of available binding data on TNFSF ligand-TNFRSF receptor interactions are gained with cell-free methods that are based on the usage of distinct experimental methods and recombinant receptor variants containing varying numbers of receptor molecules (Table 1). Therefore, it is not really unexpected that affinities published for a certain ligand-receptor interaction can vary considerably (Table 1). In particular, values derived from cell free assays are often quite different from binding parameters obtained by cellular binding studies (Table 1). Presumably, this dissatisfying situation is mainly caused by the fact that the formation of TNFSF ligand-TNFRSF receptor complexes on the cell surface is controlled by several cell intrinsic factors, such as dynamic self-assembly of TNFRSF receptors in the absence of ligand and interactions with the cytoskeleton that cannot be adequately gathered by cell-free techniques (8).

Here, we generated highly bioluminescent and functionally not compromised ligand variants of all TNFSF members by N-terminal fusion with the luciferase from *Gaussia princeps* (GpL). By help of these fusion proteins, we performed a systematic and comprehensive study on the interactions between TNFSF ligands and their cell surface-expressed receptors.

Experimental Procedures

Cloning, Production, and Purification of Recombinant Proteins—Cloning of GpL-FLAG-TNC-TNF, GpL-FLAG-TNC-TWEAK, and GpL-FLAG-TNC-CD95L has been described elsewhere (9, 10). The expression plasmids encoding the GpL-FLAG-TNC variants of the other TNF ligands used in this study have been obtained by exchange of the TNF-encoding fragment of the pCR3-based GpL-FLAG-TNC-TNF expression plasmid with PCR amplicons encoding THD-encompassing soluble versions of the other TNFSF ligand types (Table 2) by help of flanking EcoRI (5' end) and XbaI (3' end) restriction sites. A single synthetic DNA fragment encoding a QLGGGS linker followed by a FLAG epitope and an LT α protomer (amino acids 35–205) connected with an LT β protomer (amino acids 76–244) between two (GGGS)₄ linkers was used to replace the FLAG-TNC part of GpL-FLAG-TNC-LT β encoding amino acids 76–244 of LT β to obtain the expression plasmid encoding GpL-FLAG-scLT $\alpha\beta_2$. The single chain-encoded LT $\alpha\beta_2$ (scLT $\alpha\beta_2$) part has been designed in analogy to single chain-encoded constructs for TNF (11) and is largely similar to a C-terminally His-tagged scLT $\alpha\beta_2$ variant encoding LT α amino acids 62–205 and LT β amino acids 87–243 sepa-

rated by short GGSG linkers that have been published elsewhere (12). The conventional FLAG-TNC-TNFSF ligand expression constructs have been obtained by replacement of the TNF domain in the FLAG-TNC-TNF-encoding expression plasmid with THD-encompassing DNA fragments/amplicons. FLAG-scLT $\alpha\beta_2$ was obtained by replacing the FLAG-TNC-TNF cassette with the FLAG-scLT $\alpha\beta_2$ cassette of GpL-FLAG-scLT $\alpha\beta_2$. HEK293 cells transiently or stably transfected with expression plasmids encoding the recombinant protein of interest were grown near confluency and were then cultivated for 5–7 days in low serum medium (0.5–2%). Supernatants containing the recombinant proteins were collected and cleared by centrifugation and then either used directly for experiments or subjected to anti-FLAG affinity purification.

SDS-PAGE, Silver Staining, and Western Blot Analysis—For evaluation of the purity and protomer size of recombinant soluble TNFSF ligands, the protein samples were separated on 12.5% (w/v) polyacrylamide gels according to Laemmli. To visualize the proteins, the gels were then subjected to silver staining by help of a commercially available kit (Pierce® silver stain kit, Thermo Scientific, Braunschweig, Germany). For Western blot analysis of p100 to p52 processing, cells were washed once with PBS and scraped into PBS using a rubber policeman. Cells were pelleted and resuspended in 4 \times Laemmli sample buffer ($\sim 1 \times 10^6$ cells/100 μ l of buffer) supplemented with complete protease inhibitor (Roche Applied Science) and phosphatase inhibitor mixtures II (Sigma). To improve cell disintegration, samples were sonicated for 15 s with maximum amplitude (UP100H Ultrasonic Processor, Hielscher, Germany), heated at 95 $^{\circ}$ C for 5 min, and centrifuged for 5 min to remove residual insoluble material. Lysates were further processed by SDS-PAGE and standard Western blotting procedures. Finally, p100 processing was determined using anti-p100/p52 from Millipore (05-361), horseradish peroxidase-conjugated anti-mouse IgG (Dako), and the ECL Western blotting detection reagents and analysis system (Amersham Biosciences).

Binding Studies with GpL-TNFSF Ligand Fusion Proteins—Cells (typically 2×10^5 per well in 24-well plates) were cultured overnight to ensure adherence. For equilibrium binding experiments, the cells in half of the wells were dedicated for determination of nonspecific binding and were preincubated for 1 h at 37 $^{\circ}$ C with either an excess of the “GpL-less” variant of the soluble TNFSF ligand investigated or with an antibody blocking access to the membrane-bound TNFRSF receptor. Untreated cells (= total binding) and “blocked” cells were then pairwise incubated with the GpL-TNFSF ligand fusion protein of interest for an additional hour at 37 $^{\circ}$ C. Unbound GpL-TNFSF ligands were removed by 10 rapid washes in ice-cold PBS, and cells were scratched with a rubber policeman in 50 μ l of culture medium (0.5% FCS, penicillin/streptomycin). Cell-associated GpL activity was finally measured in black 96-well plates using a commercial *Gaussia* luciferase assay kit (New England Biolabs GmbH, Frankfurt a.M., Germany). To keep errors caused by the comparatively rapid decay of bioluminescent activity of GpL ($\sim T_{1/2} = 4$ min) below 3%, substrate/buffer solution was applied to only one column of a 96-well plate at one time, and light emission was immediately measured (1 s per sample, Lucy

TNFSF Ligand-TNFRSF Receptor Interactions

TABLE 1

Literature survey of TNFSF ligand-TNFRSF receptor affinities

The abbreviations used are as follows: CBS, cellular binding study; SPR, surface plasmon resonance; ITC, isothermal titration calorimetry; HRTF, homogenous time-resolved fluorescence; n.i., not indicated.

Interaction	K _D (nM)	Method	Ligand / Receptor	Ref.
TNF-TNFR1 (TNFSF2-TNFRSF1A)	2	CBS (4°C)	¹²⁵ I-TNF / cell surface	(37)
	2.5	CBS (4°C)	¹²⁵ I-TNF / cell surface	(38)
	0.019	CBS (37°C)	¹²⁵ I-TNF / cell surface	(17)
	0.65	ELISA (37°C)	¹²⁵ I-TNF / plastic-bound TNFR1-Fc	(39)
TNF-TNFR2 (TNFSF2-TNFRSF1B)	0.42	CBS (37°C)	¹²⁵ I-TNF / cell surface	(17)
LTα-TNFR1 (TNFSF1-TNFRSF1A)	0.064	ELISA (37°C)	¹²⁵ I-LTα / plastic-bound TNFR1-Fc	(39)
CD95L-CD95 (TNFSF6-TNFRSF6)	1.1	ELISA (n.i.)	Flag-CD95L / plastic-bound CD95-Fc	(40)
CD95L-DcR3 (TNFSF6-TNFRSF6B)	0.8	ELISA (n.i.)	Flag-CD95L / plastic-bound DcR3-Fc	(40)
	271	SPR (25°C)	chip-bound CD95L / DcR3-CRDs	(41)
CD40L-CD40 (TNFSF5-TNFRSF5)	0.5	CBS (4°C)	cell surface / ¹²⁵ I-CD40-Fc	(42)
41BBL-41BB (TNFSF9-TNFRSF9)	1.4	CBS (4°C)	cell surface / soluble 41BBed-AP ^a	(43)
	55.2	SPR (25°C)	Flag-41BBL / chip-bound 41BB-Fc	(44)
	1.2	SPR (25°C)	Avi-41BBL / chip-bound 41BB-Fc	(44)
	1/66 ^b 0.6/0.3	CBS (4°C)	cell surface / ¹²⁵ I-41BB-Fc	(45)
OX40L-OX40 (TNFSF4-TNFRSF4)	0.2-0.4	CBS (n.i.)	¹²⁵ I-sCD4-OX40L / cell surface	(46)
	3.8	SPR (25°C)	sCD4-OX40L / chip-bound OX40ed	(46)
	180	SPR (25°C)	chip-bound sCD4-OX40L / sOX40ed	(46)
	0.25	HTRF (RT)	Flag-sOX40L / OX40ed-Fc	(47)
CD30L-CD30 (TNFSF8-TNFRSF8)	23	CBS (n.i.)	¹²⁵ I-CD4-CD30L / cell surface	(48)
	4.5	SPR (25°C)	chip-bound CD4-CD30L / CD30-CD4	(48)
	1.1	SPR (25°C)	chip-bound CD4-CD30L / CD30-Fc	(48)
	3	CBS (4°C)	cell surface / ¹²⁵ I-CD30-Fc	(49)
LIGHT-HVEM (TNFSF14-TNFRSF14)	44	SPR (25°C)	sLIGHT / chip-bound HVEM-Fc	(50)
	1.5	SPR (n.i.)	sLIGHT / chip-bound HVEM-Fc	(51)
LIGHT-LTβR (TNFSF14-TNFRSF3)	1.3	SPR (n.i.)	sLIGHT / chip-bound LTβR-Fc	(51)
	126	ITC (25°C)	sLIGHT / LTβR	(52)
	40	SPR (4°C) ^c	sLIGHT-LTβR / chip-bound LTβR-Fc	(52)
	< 0.1	SPR (4°C) ^c	sLIGHT-LTβR-Fc / chip-bound LTβR-Fc	(52)
LIGHT-DcR3 (TNFSF14-TNFRSF6B)	0.6	SPR (n.i.)	sLIGHT / chip-bound DcR3-Fc	(51)
	14	SPR (25°C)	sLIGHT / chip-bound DcR3-Fc	(41)
LTαβ ₂ -LTβR (TNFSF1/3-TNFRSF3)	39	SPR ³ (4°C)	LTαβ ₂ -LTβR / chip-bound LTβR-Fc	(52)
	< 0.1	SPR ³ (4°C)	LTαβ ₂ -LTβRed-Fc / chip-bound LTβR-Fc	(52)
RANKL-RANK (TNFSF11-TNFRSF11A)	2090	SPR (n.i.)	chip-bound RANKL / monomeric sRANK	(53)

TABLE 1—continued

RANKL-OPG (TNFSF11-TNFRSF11B)	4.2	SPR (n.i.)	chip-bound RANKL / monomeric OPG	(53)
	6.7	SPR (25°C)	soluble RANKL / chip-bound OPG-Fc	(54)
	1.7 / 6	SPR (n.i.)	soluble RANKL / chip-bound OPG-Fc	(55)
	8.4 23	SPR (20°C) SPR (37°C)	sRANKL / chip-bound OPG	(56)
	6.4 12.5	SPR (20°C) SPR (37°C)	sRANKL / chip-bound OPG-Fc	(56)
TRAIL-OPG (TNFSF10-TNFRSF11B)	41 463	SPR (20°C) SPR (37°C)	sTRAIL / chip-bound OPG	(56)
	45 46	SPR (20°C) SPR (37°C)	sTRAIL / chip-bound OPG-Fc	(56)
	3	SPR (25°C)	Flag-sTRAIL / chip-bound OPG-Fc	(57)
	400	ITC (37°C)	sTRAIL / OPG-Fc	(58)
TL1A-DeR3 (TNFSF15-TNFRSF6B)	56	SPR (25°C)	chip-bound TL1A / DeR3-CRDs	(41)
TL1A-DR3 (TNFSF15-TNFRSF12)	4.6	CBS (37°C)	GpL-TL1A fusion protein / cell-expressed death domain deletion mutant of DR3	(59)
TRAIL-TRAILR1 (TNFSF10-TNFRSF10A)	70	ITC (37°C)	sTRAIL / TRAILR1-Fc	(58)
TRAIL-TRAILR2 (TNFSF10-TNFRSF10B)	2	ITC (37°C)	sTRAIL / TRAILR2-Fc	(58)
	0.76	SPR (25°C)	Flag-sTRAIL / chip-bound TRAILR2-Fc	(57)
TRAIL-DeR1 (TNFSF10-TNFRSF10C)	200	ITC (37°C)	sTRAIL / TRAILR3-Fc	(58)
	1.1	SPR (25°C)	Flag-sTRAIL / chip-bound TRAILR3-Fc	(57)
TRAIL-TRAILR4 (TNFSF10-TNFRSF10D)	5.5	HTRF (RT)	sTRAIL / TRAILR4-Fc	(47)
BAFF to B-cells	0.1 – 1.1	CBS (RT)	¹²⁵ I-Baff / cell surface	(60)
BAFF-BAFFR (TNFSF13B-TNFRSF13C)	16	SPR (n.i.)	chip-bound Myc-Baff / monomeric sBAFFR	(61)
	0.1	ELISA (37°C)	Myc-Baff / plastic-bound BAFFR-Fc	(61)
BAFF-BCMA (TNFSF13B-TNFRSF17)	1550	SPR (n.i.)	chip-bound Myc-Baff / monomeric sBCMA	(61)
	0.15	ELISA (37°C)	Myc-Baff plastic-bound BCMA-Fc	(61)
	4.9	SPR (n.i.)	chip-bound Baff / BCMA-Fc	(62)
BAFF-TACI (TNFSF13B-TNFRSF13B)	0.38	CBS (RT)	¹²⁵ I-Baff / TACI transfectants	(63)
	0.16	SPR (n.i.)	BAFF / chip-bound TACI-Fc	(63)
APRIL-BAFFR (TNFSF13-TNFRSF13C)	> 3000	SPR (n.i.)	chip-bound Flag-APRIL / monomeric sBAFFR	(61)
APRIL-BCMA (TNFSF13-TNFRSF17)	16	SPR (n.i.)	chip-bound Flag-APRIL / monomeric sBCMA	(61)
	5.5	SPR (n.i.)	chip-bound APRIL / sBCMA-Z	(62)
	0.2	SPR (n.i.)	chip-bound APRIL / sBCMA-Fc	(62)
APRIL-TACI (TNFSF13-TNFRSF13B)	6.4	SPR (n.i.)	APRIL / chip-bound TACI-Fc	(63)
TWEAK-Fn14 (TNFSF12-TNFRSF12A)	2.3	CBS (4°C)	¹²⁵ I-Fn14-Fc / TWEAK transfectants	(64)
	0.8-4.7	SPR (n.i.)	Fn14-Fc / chip-bound TWEAK	(65)
GITRL-GITR (TNFSF18-TNFRSF18)	400-700	SPR (25°C)	sGITRL / chip-bound GITR	(66)
	559	SPR (25°C)	sGITRL / chip-bound GITR	(67)
	4370	SPR (25°C)	mGITR-Fc / chip-bound mGITRL	(68)

^a 41BBed-AP is a fusion protein of the extracellular domain of 41BB with alkaline phosphatase, and the latter forms dimers.

^b Endogenous/transfected 41BBL of high and low affinity binding sites.

^c Solution phase BIAcore binding assay (for details see Ref.)

TNFSF Ligand-TNFRSF Receptor Interactions

TABLE 2

Amino acid residues of full-length TNFSF ligands contained in the soluble TNFSF ligand variants

TNFSF ligand	Accession no.	AA	Construct
TNFSF1 / LT α	NP_000586	35-205	Flag-TNC-LT α -pCR3 GpL-Flag-TNC-LT α -pCR3 Flag-LT α -LT β -LT β -pCR3 GpL-Flag-LT α -LT β -LT β -pCR3
TNFSF2 / TNF	NP_000585	85-233	Flag-TNC-TNF-pCR3 GpL-Flag-TNC-TNF-pCR3
TNFSF3 / LT β	NP_002332	76-244	Flag-LT α -LT β -LT β -pCR3 GpL-Flag-LT α -LT β -LT β -pCR3
TNFSF4 / OX40L	NP_003317	52-183	Flag-TNC-OX40L-pCR3 GpL-Flag-TNC-OX40L-pCR3
TNFSF5 / CD40L	AAH71754.1	116-261	Flag-TNC-CD40L-pCR3 GpL-Flag-TNC-CD40L-pCR3
TNFSF6 / CD95L	NP_000630	137-281	Flag-TNC-CD95L-pCR3 GpL-Flag-TNC-CD95L-pCR3
TNFSF7 / CD27L	NP_001243	52-193	Flag-TNC-CD27L-pCR3 GpL-Flag-TNC-CD27L-pCR3
TNFSF8 / CD30L	NP_001235	61-234	Flag-TNC-CD30L-pCR3 GpL-Flag-TNC-CD30L-pCR3
TNFSF9 / 4-1BBL	NP_003802	85-254	Flag-TNC-41BBL-pCR3 GpL-Flag-TNC-41BBL-pCR3
TNFSF10 / TRAIL	NP_003801	95-281	Flag-TNC-TRAIL-pCR3 GpL-Flag-TNC-TRAIL-pCR3
TNFSF11 / RANKL	AAB86811	69-316	Flag-TNC-RANKL-pCR3 GpL-Flag-TNC-RANKL-pCR3
TNFSF12 / TWEAK	BAE16557	106-249	Flag-TWEAK-pCR3 GpL-Flag-TNC-TWEAK-pCR3
TNFSF13 / APRIL	CAG46870	115-250	Flag-TNC-APRIL-pCR3 GpL-Flag-TNC-APRIL-pCR3
TNFSF13B / BAFF	NP_006564	137-285	Flag-TNC-BAFF-pCR3 GpL-Flag-TNC-BAFF-pCR3
TNFSF14 / LIGHT	NP_003798	89-240	Flag-TNC-LIGHT-pCR3 GpL-Flag-TNC-LIGHT-pCR3
TNFSF15 / TL1A	NP_005109	93-251	Flag-TNC-TL1A-pCR3 GpL-Flag-TNC-TL1A-pCR3
TNFSF18 / GITRL	NP_005083	72-199	Flag-TNC-GITRL-pCR3 GpL-Flag-TNC-GITRL-pCR3
EDA / EDA-A1	NP_001390	179-391	Flag-TNC-EDA-A1-pCR3 GpL-Flag-TNC-EDA-A1-pCR3
EDA / EDA-A2	NP_001005609	179-391	Flag-TNC-EDA-A2-pCR3 GpL-Flag-TNC-EDA-A2-pCR3
Murine TNFSF2 / TNF	NP_038721	89-235	GpL-Flag-TNC-muTNF-pCR3
Murine TNFSF7 / CD27L	NP_035747	54-195	GpL-Flag-TNC-muCD27L-pCR3
Murine TNFSF11 / RANKL	NP_035743	72-316	GpL-Flag-TNC-muRANKL-pCR3
Murine TNFSF12 / TWEAK	NP_035744	106-249	GpL-Flag-TNC-muTWEAK-pCR3

2 Luminometer, Anthos Labtec Instruments, Krefeld, Germany). In the cases where TNFRSF receptor transfectants have been used for the determination of total binding, the corresponding parental and receptor-negative transfectants have been used for the evaluation of nonspecific binding without further “blocking” treatment.

TNFSF Ligand-induced Apoptosis and IL8 Production—To evaluate the activity of soluble TNFSF ligands and GpL-TNFSF ligand fusion proteins, HT1080 cells, or HT1080 transfectants of the TNFRSF of interest, were seeded in 96-well tissue culture plates (2×10^4 /well) and cultured overnight. The following day, cells were stimulated in triplicate with the recombinant ligands. In experiments where oligomerized ligand molecules have been used, ligands were preincubated on a separate 96-well plate with 1 μ g/ml FLAG-specific mAb M2 irrespective of the ligand concentration and were transferred to the cells after 15–30 min. In cases where IL8 induction has been investigated, medium was exchanged prior to ligand stimulation to minimize the background of constitutively produced IL8. Next day, IL8 production and cellular viability were measured. In cell death experiments, cells were sensitized for apoptosis induction by

co-treatment with 2.5 μ g/ml cycloheximide. IL8 in cell culture supernatants was quantified using a commercially available ELISA kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer’s instructions, and cellular viability was quantified by crystal violet staining.

Results

Generation, Production, and Purification of a Panel of Soluble GpL Fusion Proteins Covering the Human Members of the TNFSF Ligand Family—A variety of studies with soluble fusion proteins containing the THD of TNFSF ligands suggest that the functionality of the THD with respect to receptor binding and receptor activation is largely insensitive to N-terminal linkage of additional protein domains (3). We recently took advantage of this fact and generated GpL fusion proteins of the TNFSF ligands CD95L, TNF, and TWEAK for cellular binding studies (9, 10). These GpL-TNFSF ligand fusion proteins were proved to be highly traceable and functionally not distinguishable from their conventional counterparts. We extended this work and generated a panel of GpL fusion protein covering all human members of the TNFSF ligand family for the systematic and comprehensive analysis of TNFSF ligand-TNFRSF receptor interactions on intact cells (Fig. 1, A and B). The GpL-TNFSF ligand fusion proteins consist of an N-terminal GpL domain and C-terminally of the THD of the various human TNFSF ligands. To facilitate purification of the GpL-TNFSF ligand fusion proteins, we furthermore introduced an internal FLAG epitope between the GpL and TNFSF ligand domain. Soluble TNFSF ligands typically assemble with high efficiency into trimeric molecules and interact with members of the TNFRSF receptors. However, in some cases soluble TNF ligand variants are unstable and tend to form not only trimers but to a varying extent inactive misfolded aggregates (13, 14). We and others have previously found that the formation of the latter can be reduced by introduction of a heterologous trimerization domain, such as a modified leucine zipper or the tenascin-C trimerization domain (13–15). Therefore, we also introduced the trimerization domain of TNC preceding the THD to ensure proper production and trimer assembly of all TNFSF ligands used in this study (Fig. 1A, left panel). To obtain LT α -LT β heterotrimers of defined 1:2 stoichiometry, we encoded peptide linker-connected LT α and LT β protomers (single-chain LT $\alpha\beta_2$, scLT $\alpha\beta_2$) by a single DNA expression cassette (Fig. 1B, right panel). Supernatants collected from HEK293 cells transiently transfected with expression plasmids encoding the various GpL-FLAG-TNC-TNFSF ligand fusion proteins typically yielded 30–120 μ g of recombinant protein per 15-cm cell culture Petri dish. GpL-FLAG-TNC-TRAIL showed the poorest productivity and only yielded ~ 10 μ g per 15-cm cell culture Petri dish despite supplementing the culture medium with zinc ions, which are required by this TNF ligand for proper folding (16). SDS-PAGE and silver staining of anti-FLAG affinity chromatography-purified GpL-FLAG-TNC-TNFSF ligand fusion proteins revealed one to three bands that correspond in size to the deduced molecular weight of the non-modified ligand molecules or to moderately glycosylated forms (Fig. 1B). Luciferase activity of all GpL-FLAG-TNC-TNFSF ligand fusion proteins was between 940 and 1360 relative light units per s and fmol.

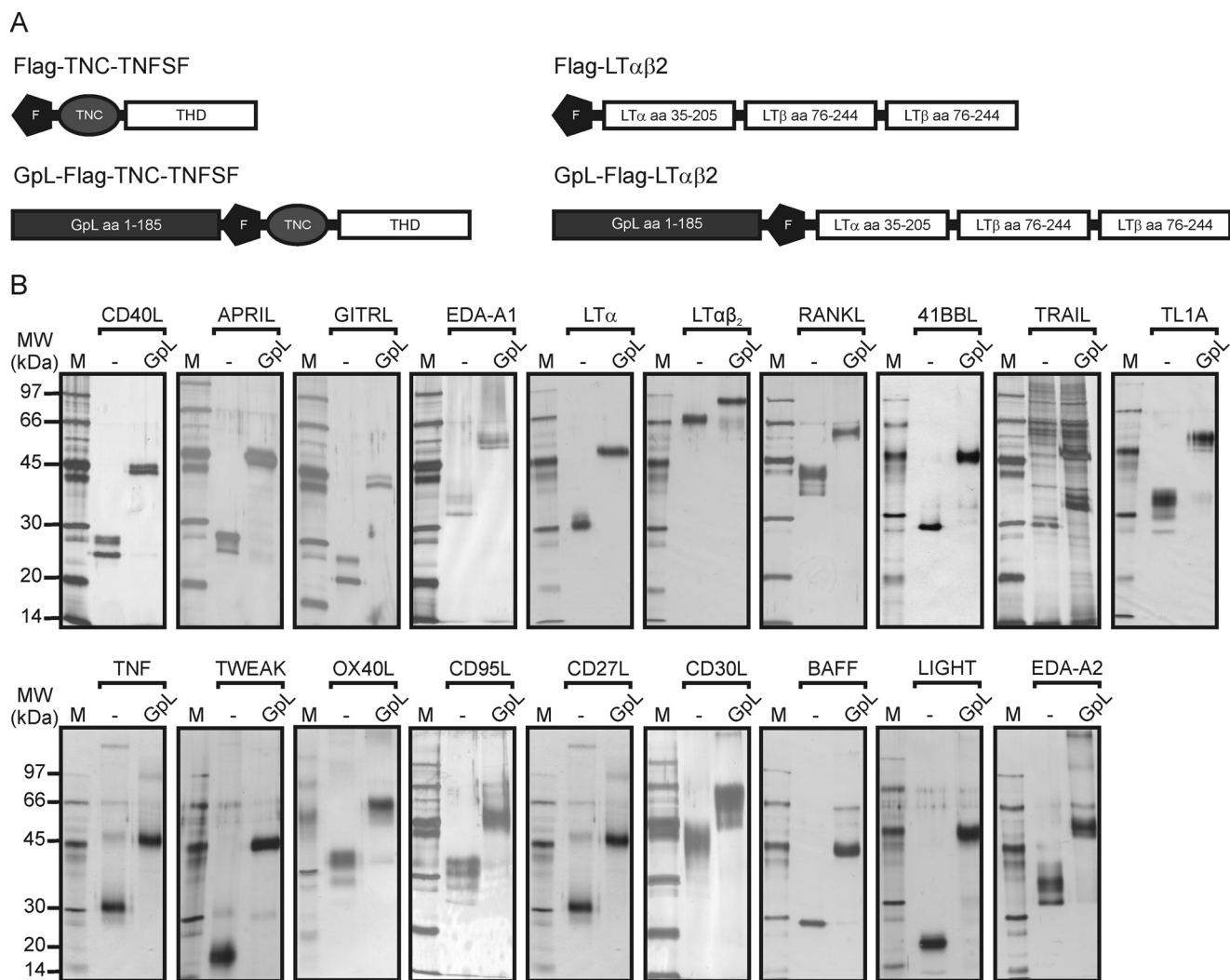


FIGURE 1. Structure and purification of GpL-TNFSF ligand fusion proteins. *A*, general domain architecture of the soluble TNFSF ligand variants used in this study. *Left panel*, TNC domain-stabilized soluble TNFSF ligands with and without the GpL domain. *Right panel*, single-chain encoded LT $\alpha\beta$ 2 heterotrimeric molecules. *F*, FLAG epitope; *TNC*, trimerization domain of tenascin-C. *B*, 100 ng of the indicated anti-FLAG M2-agarose affinity-purified TNFSF ligands were separated by SDS-PAGE and visualized by silver staining. *Lane M*, molecular weight marker.

Thus, there was no evidence for a TNFSF ligand-specific impact of the THD in the various GpL-FLAG-TNC-TNFSF ligand fusion proteins on the activity of the GpL domain. As found before for the GpL fusion proteins of CD95L, luciferase activity increased linearly over several orders of magnitude with the concentration of the GpL-FLAG-TNC-TNFSF ligand fusion proteins (data not shown).

GpL Fusion Proteins of Soluble TNFSF Ligands Display Unaltered Receptor Stimulating Activities—Next, we investigated for a subset of the novel GpL-FLAG-TNC-TNFSF ligands whether the GpL domain interferes with the receptor stimulating activity of the THD contained in these molecules. For this purpose, we determined side-by-side the dose dependence of induction of apoptosis, IL8 production, or p100 processing by the GpL fusion proteins of 4-1BBL, CD27L, OX40L, CD40L, GITRL, TRAIL, TNF, CD95L, LT α , LIGHT, and scLT $\alpha\beta$ 2 and the corresponding conventional ligands without GpL domain. We observed in no case major differences in the corresponding ED₅₀ values between the GpL domain-containing and the GpL domain-less variants (Figs. 2 and 3). For example, GpL-FLAG-

TNC-TRAIL induced apoptosis in HT1080 cells with an ED₅₀ value of 250 pM (= 20 ng/ml), whereas conventional FLAG-TNC-TRAIL showed an ED₅₀ value of 43 pM (= 6 ng/ml) (Fig. 2A). Likewise, GpL-FLAG-TNC-LT α , GpL-FLAG-TNC-GITRL, GpL-FLAG-TNC-CD40L, GpL-FLAG-TNC-41BBL, GpL-FLAG-TNC-CD95L, GpL-FLAG-TNC-OX40L, GpL-FLAG-TNC-CD27L, and GpL-FLAG-TNC-LIGHT triggered IL8 production in HT1080 cells with comparable efficacy as their GpL domain-less counterparts (Fig. 2B). Induction of p100 processing by GpL-FLAG-TNC-CD27L, GpL-FLAG-TNC-LIGHT, GpL-FLAG-TNC-OX40L, GpL-FLAG-TNC-CD40L, and GpL-FLAG-scLT $\alpha\beta$ 2, on the one hand, and FLAG-TNC-CD27L, FLAG-TNC-LIGHT, FLAG-TNC-OX40L, FLAG-TNC-CD40L, and FLAG-scLT $\alpha\beta$ 2, on the other hand, also occurred with similar efficacy (Fig. 3). It is well established that some members of the TNF receptor superfamily require oligomerization of their corresponding soluble trimeric ligands to become properly activated. Importantly, the presence of a GpL domain had no influence or only a minor influence on this requirement (Figs. 2 and 3). Together, the dose-response anal-

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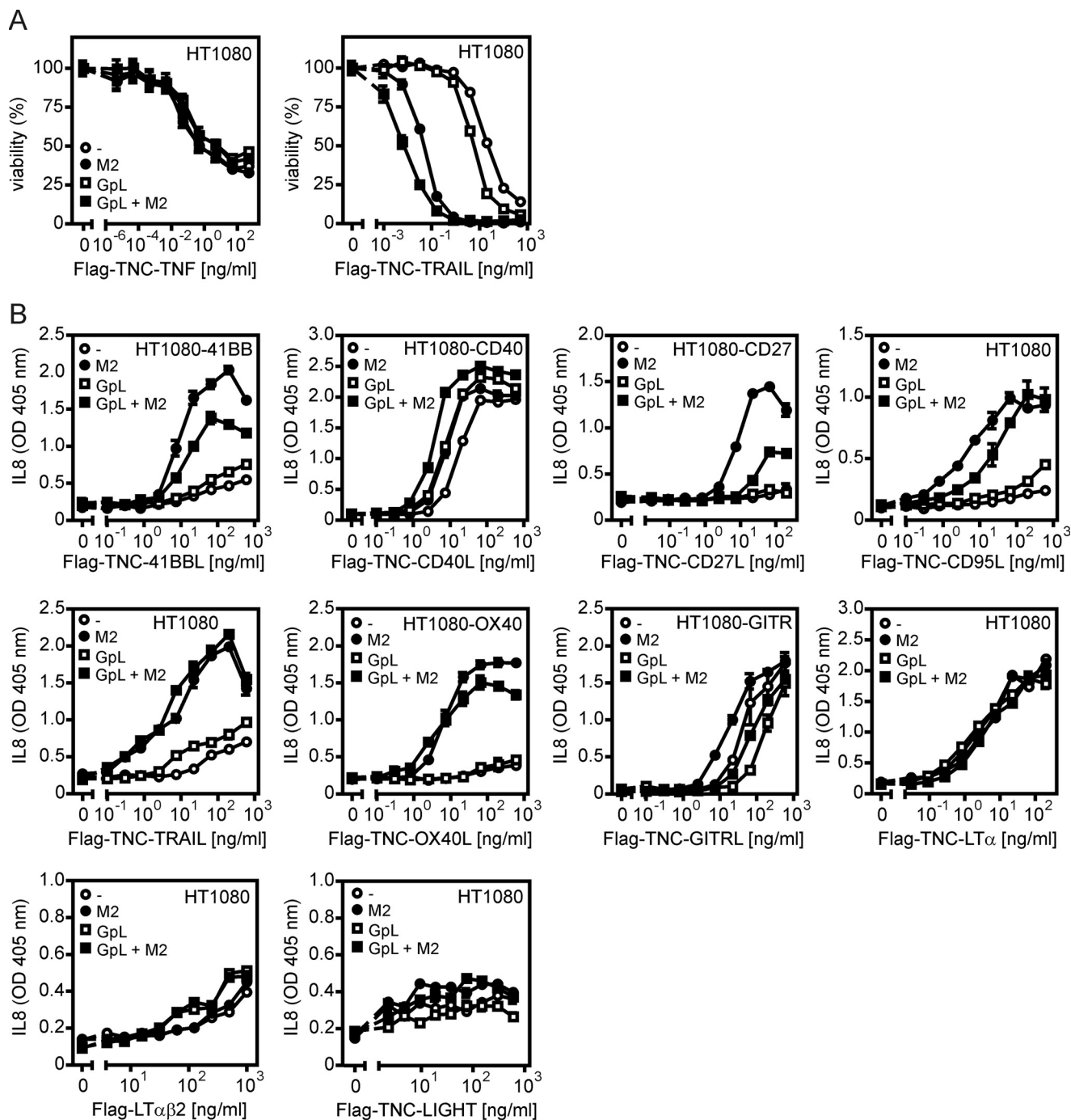


FIGURE 2. Induction of apoptosis and IL8 production by GpL-TNFSF ligand fusion proteins and conventional soluble TNFSF ligands. *A*, HT1080 cells were grown in 96-well plates (20,000 cells per well) overnight, sensitized for apoptosis by treatment with 2.5 μ g/ml cycloheximide and then challenged for an additional day with the indicated concentrations of GpL-FLAG-TNC-TRAIL and GpL-FLAG-TNC-TNF in the presence and absence of 1 μ g/ml anti-FLAG mAb M2. Cell viability was finally quantified by crystal violet staining. *B*, HT1080 cells (for analysis of CD95L, TRAIL, LIGHT, LT α , and LT α β ₂) and HT1080 transfectants expressing 4-1BB, CD27, GITR, CD40, or OX40 were seeded in 96-well plates (10,000 cells per well). The next day, medium was changed to reduce the background of constitutively produced IL8, and cells were then challenged overnight in triplicate with increasing concentrations of the indicated TNFSF ligand variants in the absence and presence of 1 μ g/ml of the FLAG-specific mAb M2. Finally, the IL8 content of supernatants was determined by ELISA analysis.

ysis experiments indicate that genetic fusion of the GpL domain to the N-terminal end of the THD of soluble TNFSF ligands has no major impact on the TNFSF ligand-TNFRSF receptor interaction. Thus, N-terminal fusion of the GpL domain is a generally applicable strategy for labeling soluble TNFSF ligands for studies on TNFSF ligand-TNFRSF receptor interactions in living cells.

Binding Studies with GpL-FLAG-TNC-tagged TNFSF Ligand Fusion Proteins—To determine binding affinities of the various TNFSF ligands at 37 °C to their cell-expressed TNFRSF receptors, saturation binding studies were performed using the various GpL-TNFSF ligand fusion proteins. Depending on the particular TNFRSF receptor-TNFSF ligand pair that has been investigated, nonspecific binding was determined in two differ-

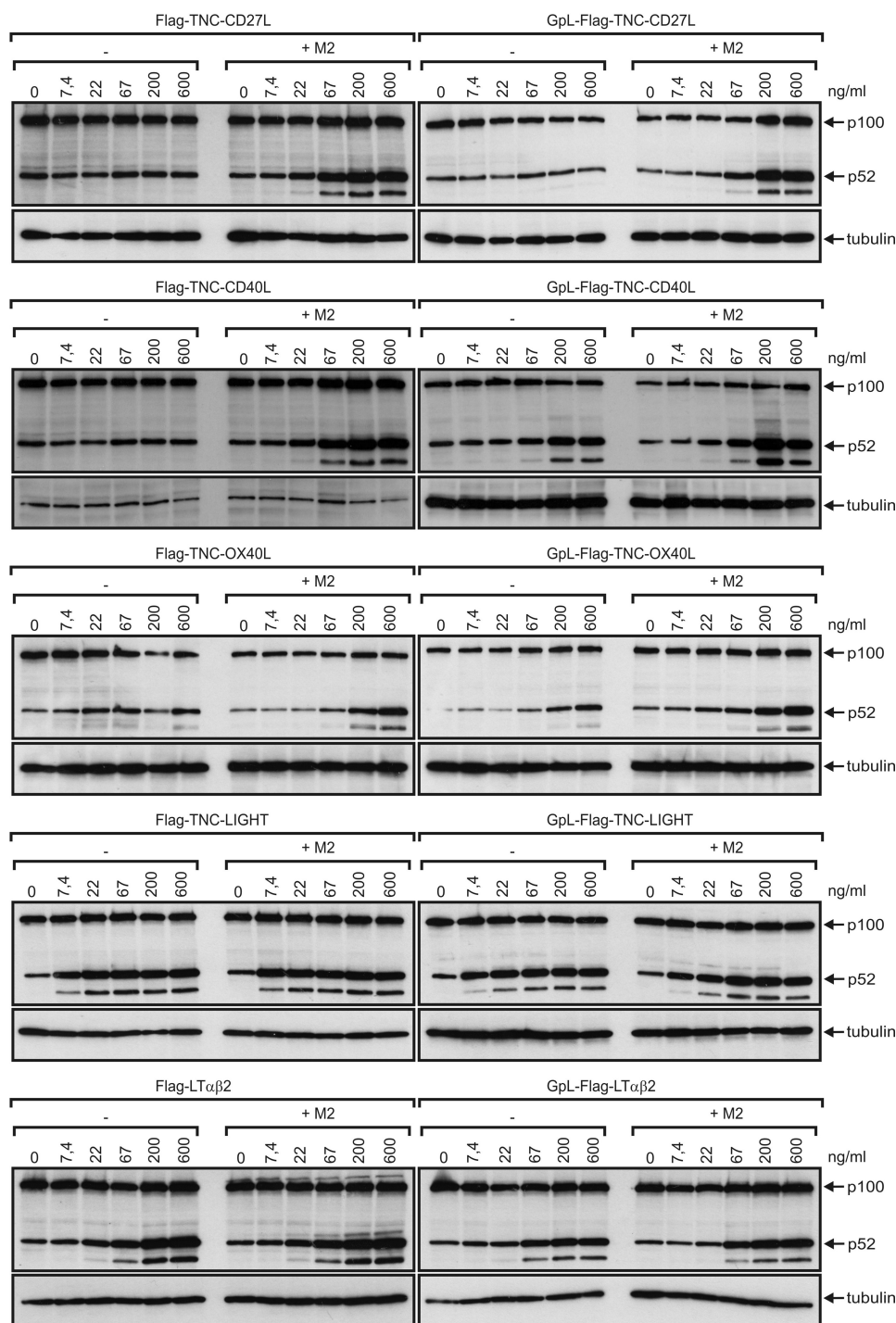


FIGURE 3. Induction of p100 processing by GpL-TNFSF ligand fusion proteins and conventional soluble TNFSF ligands. Cells (HT1080 for LIGHT and $LT\alpha\beta_2$, HT1080 TNFRSF receptor transfectants for CD40L, CD27L, and OX40L) were challenged with increasing concentrations of the indicated TNFSF ligands in the presence and absence of the anti-FLAG mAb M2 (1 μ g/ml). After overnight incubation, total cell lysates were prepared and subjected to Western blot analysis to evaluate p100 to p52 expression.

ent ways. For a few TNF receptors (CD40, TNFR2, CD27, OX40, 41BB, GITR, and CD30) that are not endogenously expressed in HT1080 or HeLa cells, we had available stable transfectants from other work. In these cases, specific binding was calculated as the difference of total binding of a GpL-TNFSF ligand fusion protein to the corresponding receptor-expressing transfectant and its nonspecific binding to the receptor expression-negative parental cell line. Similarly, bind-

ing studies with GpL-FLAG-TNC-BAFF, GpL-FLAG-TNC-APRIL, GpL-FLAG-TNC-EDA-A2, GpL-FLAG-TNC-TL1A, and GpL-FLAG-TNC-TRAIL were performed using HEK293 cells transiently transfected with expression vectors encoding BCMA, BAFFR, TACI, XEDAR, DR3, and the TRAIL receptors (TRAILR1, TRAILR2, TRAILR3, and TRAILR4) and mock-transfected control cells. HEK293 cells express moderate amounts of TRAIL receptors, but this barely affected the bind-

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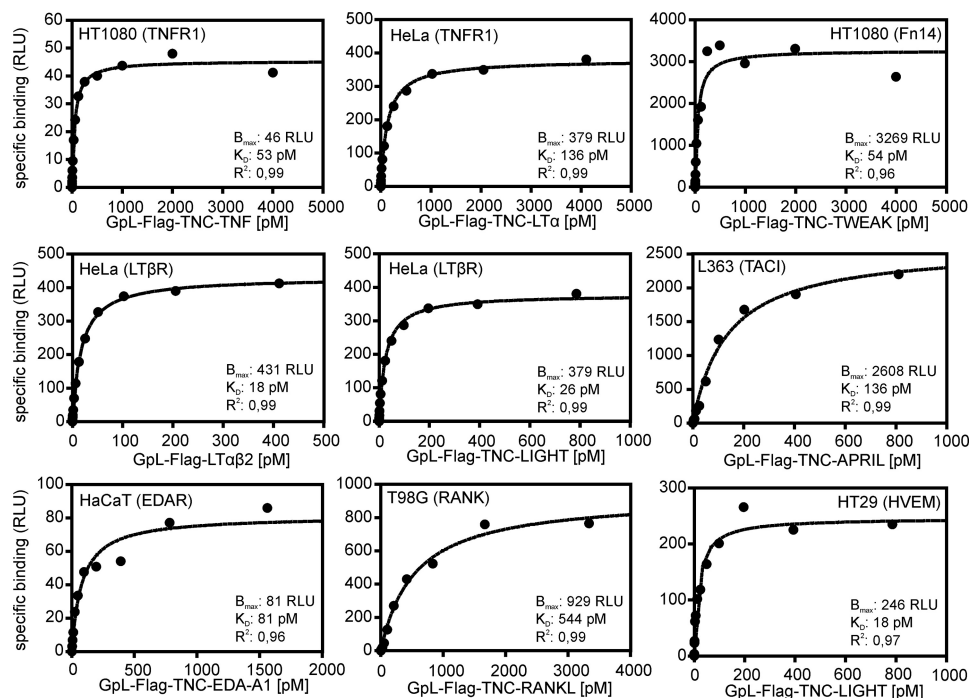


FIGURE 4. Saturation binding studies with GpL-TNFSF ligand fusion proteins and endogenously expressed TNFRSF receptors. Cells of the indicated cell lines (2×10^5 cells/well) were cultivated overnight in 24-well plates. The next day, half of the wells were preincubated with an excess ($2 \mu\text{g/ml}$) of the “GpL-less” soluble TNFSF ligand or in the case of the interaction of TNF and $\text{LT}\alpha$ with TNFR1 with the mAb H398 ($10 \mu\text{g/ml}$) to block access to the membrane-bound TNFRSF receptor. Untreated cells (= total binding) and blocked cells (= nonspecific binding) were then pairwise incubated with the GpL-TNFSF ligand fusion proteins for an additional hour at 37°C . After removal of unbound molecules, the nonspecific binding values were subtracted from the corresponding total binding values to obtain specific binding values that were fitted by non-linear regression to a single binding site interaction plot using the GraphPad Prism5 software. One representative experiment is shown for each of the TNFSF ligand-TNFRSF receptor interactions. For the number of experiments and statistics of each interaction, see Table 3.

ing studies due to the much higher number of ectopically expressed TRAIL receptors. In the remaining cases, nonspecific binding was determined by preincubating cells either with an excess of the GpL-less ligand variant or with a blocking TNFRSF receptor-specific antibody. Specific binding was then calculated again by subtracting the nonspecific binding from total binding. The affinities obtained ranged over 3 orders of magnitude reaching from low concentrations of 0.010 and 0.017 nM for $\text{LT}\alpha\beta_2$ and LIGHT for binding to the $\text{LT}\beta\text{R}$ to concentrations of 7.1 and 19.2 nM for the CD40L-CD40 interaction and the CD30L-CD30 interaction (Figs. 4 and 5 and Table 3).

In view of the great relevance of mouse models for basic and preclinical research in the TNFSF/TNFRSF field, we also spot-checked a couple of murine TNFSF ligand-TNFRSF receptor interactions. In all five interactions investigated, there was no difference or only a very minor difference in the affinity of the human and murine ligand-receptor pair (Fig. 6 and Table 4). This suggests that not only the specificities of the TNFSF ligand-TNFRSF receptor interactions are largely conserved between mouse and men but also the strength of these interactions.

Discussion

Although soluble TNFSF ligand variants comprising the THD typically retain the capability to interact with TNFRSF receptors, in several cases this is not sufficient to ensure robust receptor activation. Noteworthy, this seems not to reflect an intrinsic insufficiency of the soluble ligand molecules but rather

defines different requirements of TNFRSF receptors for the way the ligands are presented. This is evident from the interactions of TNF with TNFR1 and TNFR2 as well as from studies with APRIL and the receptors TACI and BAFFR. Soluble TNF interacts efficiently with TNFR1 and TNFR2 (Tables 1 and 3) (17), but only TNFR1 becomes strongly activated this way, although proper TNFR2 activation requires stimulation by membrane TNF (18, 19). Likewise, activation of TACI occurs in response to proteoglycan-attached APRIL but not upon binding of soluble APRIL lacking the proteoglycan-binding site of the molecule (20, 21). There are several other TNFRSF receptors that despite ligand binding are not or are only poorly activated by soluble ligand trimers, *e.g.* CD95, TRAILR2, OX40, CD27, 41BB, and GITR (14, 22–24). A latent capability to stimulate strong receptor signaling, however, is also present in “poorly active” TNFSF ligand-TNFRSF receptor complexes. First, strong receptor activation by poorly active soluble ligand trimers can be restored by multimerization, *e.g.* by oligomerization with antibodies recognizing an epitope/tag not interfering with receptor binding or by genetically enforced formation of hexameric, nonameric, or dodecameric molecules (14, 19, 22, 23, 25–30). Second, inefficient receptor activation by soluble ligand trimers can be overcome by binding to the extracellular matrix or by artificial cell surface immobilization using genetically engineered trimeric variants of soluble TNF ligands (31). A quite simple and straightforward explanation for the enhanced activity of oligomerized soluble TNFSF ligands

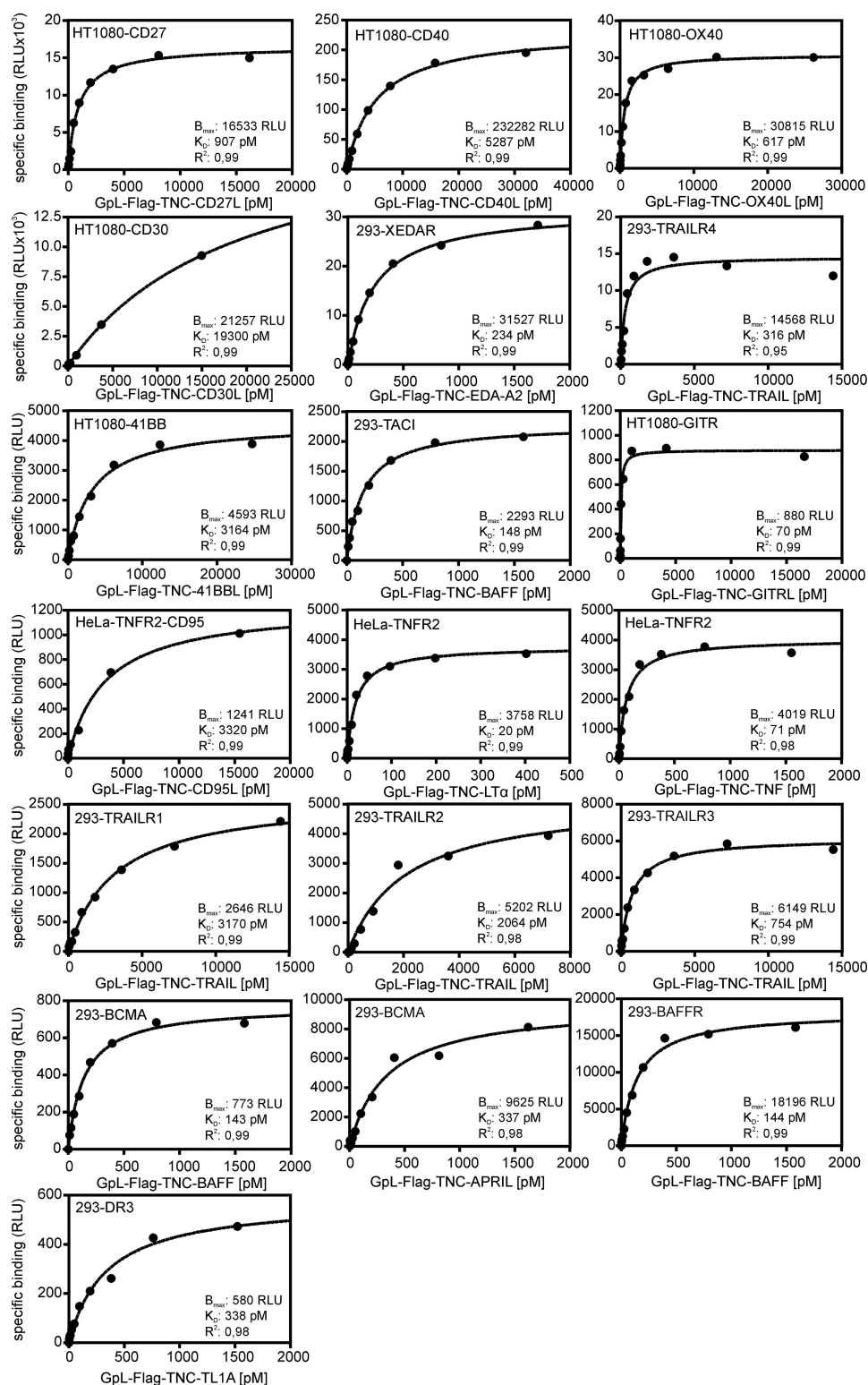


FIGURE 5. **Saturation binding studies with GpL-TNFSF ligand fusion proteins and TNFRSF receptor transfectants.** HT1080 and HeLa transfectants stably expressing the indicated TNFRSF receptors and HEK293 transiently transfected with expression plasmids encoding the indicated TNFRSF receptor along with corresponding control cells (HT1080, HeLa, and mock-transfected HEK293 cells and HeLa-TNFR2 transfectants in experiments with HeLa-TNFR2-CD95 double transfectants) were cultivated overnight in 24-well plates (half-plate control cells and half-plate receptor transfectants). The next day, cells were pairwise-incubated with increasing concentrations of the GpL-TNFSF ligand fusion proteins for an additional hour at 37 °C. After removal of unbound molecules, the nonspecific binding values (= HT1080, HeLa, and HEK293 cells and HEK293 cells and HeLa-TNFR2 transfectants in experiments with HeLa-TNFR2-CD95 double transfectants) were subtracted from the total binding values (= HT1080, HeLa, and HEK293 receptor transfectants, HeLa-TNFR2-CD95 double transfectants) to obtain specific binding values. The latter were fitted by non-linear regression to a single binding site interaction plot with the GraphPad Prism5 software. One representative experiment is shown for each of the TNFSF ligand-TNFRSF receptor interactions. For the number of experiments and statistics of each interaction, see Table 3.

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TABLE 3

TNFSF ligand-TNFRSF receptor affinities derived from cellular binding studies at 37 °C and their comparison with literature values

For references of affinity constants, see Table 1. All binding studies considered were performed at 37 °C and showed R^2 values for non-linear regression of specific binding data of >0.95.

Interaction	K_D (nM) – this study	Number of experiments	K_D (nM) – literature	Oligomerization enhancement index
TNF-TNFR1 (TNFSF2-TNFRSF1A)	0.040 ± 0.010	5	0.019 – 2.5	1; ref. (23) + Fig. 2A
TNF-TNFR2 (TNFSF2-TNFRSF1B)	0.082 ± 0.028	4	0.42	> 100; ref. (23)
LT α -TNFR1 (TNFSF1-TNFRSF1A)	0.158 ± 0.057	5	0.064	1; Fig. 2B
LT α -TNFR2 (TNFSF1-TNFRSF1B)	0.047 ± 0.01	6	-	-
CD27L-CD27 (TNFSF7-TNFRSF7)	0.801 ± 0.140	4	-	> 100; ref. (14) + Fig 2B
CD95L-CD95 (TNFSF6-TNFRSF6)	1.55 ± 0.609	4	1.1	100 - > 1000; ref. (23) + Fig. 2B
CD95L-DcR3 (TNFSF6-TNFRSF6B)	-	-	0.8 - 271	-
CD40L-CD40 (TNFSF5-TNFRSF5)	7.13 ± 4.39	3	0.5	5-20; ref. (14)
41BBL-41BB (TNFSF9-TNFRSF9)	1.81 ± 0.65	6	1.2 – 55.2	> 100; ref. (14) + Fig 2B
OX40-OX40L (TNFSF4-TNFRSF4)	1.0 ± 0.33	5	0.2 - 180	> 100; ref. (22) + Fig 2B
CD30L-CD30 (TNFSF8-TNFRSF8)	19.22 ± 5.21	3	1.1 - 23	-
LIGHT-HVEM (TNFSF14-TNFRSF14)	0.182 ± 0.104	3	1.5 - 44	-
LIGHT-LT β R (TNFSF14-TNFRSF3)	0.017 ± 0.003	4	< 0.1 - 126	1; Fig 2B, Fig. 3
LT $\alpha\beta_2$ -LT β R (TNFSF1/3-TNFRSF3)	0.0096 ± 0.0017	7	< 0.1 - 39	1; Fig 2B, Fig. 3
LIGHT-DcR3 (TNFSF14-TNFRSF6B)	-	-	0.6	-
RANKL-RANK (TNFSF11-TNFRSF11A)	0.337 ± 0.048	8	2090	-
RANKL-OPG (TNFSF11-TNFRSF11B)	-	-	0.65 - 23	-
TRAIL-OPG (TNFSF10-TNFRSF11B)	-	-	3 - 463	-
TL1A-DcR3 (TNFSF15-TNFRSF6B)	-	-	56	-
TL1A-DR3 (TNFSF15-TNFRSF12)	0.134 ± 0.069	4	4.6 ^a	1 to 4; ref. (59)

TABLE 3—continued

Interaction	K_D (nM) – this study	Number of experiments	K_D (nM) – literature	Oligomerization enhancement index
TRAIL-TRAILR1 (TNFSF10-TNFRSF10A)	4.08 ± 0.5^b	3	70	100; ref. (69)
TRAIL-TRAILR2 (TNFSF10-TNFRSF10B)	1.1 ± 0.48^b	3	0.76 - 2	100 - > 1000; ref. (23,69)
TRAIL-DcR1 (TNFSF10-TNFRSF10C)	3.68 ± 3.01^b	3	1.1 - 200	-
TRAIL-TRAILR4 (TNFSF10-TNFRSF10D)	0.869 ± 0.430^b	3	5.5	-
BAFF-BAFFR (TNFSF13B-TNFRSF13C)	0.134 ± 0.008	3	0.1 - 16	1; ref. (34)
BAFF-BCMA ^c (TNFSF13B-TNFRSF17)	0.223 ± 0.065	3	0.15 – 1550	100 - > 1000; ref. (34)
BAFF-TACI (TNFSF13B-TNFRSF13B)	0.265 ± 0.059	3	0.16 - 0.38	-
APRIL-BAFFR (TNFSF13-TNFRSF13C)	-	-	> 3000	-
APRIL-BCMA (TNFSF13-TNFRSF17)	0.468 ± 0.079	3	0.2 - 16	-
APRIL-TACI (TNFSF13-TNFRSF13B)	0.236 ± 0.049	5	6.4	50 - > 100; ref. (21,34)
TWEAK-Fn14 ^d (TNFSF12-TNFRSF12A)	0.152 ± 0.05	3	0.8-4.7	> 100; ref. (70)
GITRL-GITR (TNFSF18-TNFRSF18)	0.061 ± 0.019	4	400 - 4370	~5; ref. (14)
EDAA1-EDAR ^e	0.05 ± 0.016	3	-	100 -1000; ref. (33)
EDAA2- XEDAR	0.376 ± 0.14	5	-	-

^a In this study, binding to full-length DR3 has been determined, and in the cited study binding to a death domain deletion mutant of DR3 has been investigated.

^b Our GpL-FLAG-TNC-TRAIL preparation contained significant impurities (see Fig. 1). Folding, integrity, and thus specific activity of recombinant TRAIL preparations differ notoriously, depending on the process of production and purification. The affinities of soluble GpL-TNC-FLAG-TRAIL for the various cell-expressed TRAIL receptors indicated here could therefore be even higher.

^c Functional data have been acquired with transfectants expressing an artificial BCMA-CD95 chimeric receptor.

^d The enhancing effect observed in this study depends on the TWEAK-induced pathway considered. Oligomerization lowered the EC_{50} value for classical NF- κ B signaling for 2 orders of magnitude and more but had no effect on triggering p100 processing.

^e Activity data were obtained with a soluble trimeric EDA-A1 variant without the oligomerizing collagen domain of this molecule and transfectants expressing an artificial EDAR-CD95 chimeric receptor.

would be that the increase in avidity that is associated with the oligomerization process results in a higher apparent affinity that compensates for low affinity of certain TNFSF-TNFRSF interactions. Similarly, membrane anchoring might compensate for low affinities by reducing ligand mobility and increasing local ligand concentrations. However, this view is not supported by the affinities we found for the various TNFSF ligand-TNFRSF receptor interactions. Our set of data shows no correlation between the affinities of the TNFSF ligand-TNFRSF receptor interactions and the activity-enhancing effect of ligand oligomerization. For example, TNF binds with high affinities of 0.04 and 0.082 nM to TNFR1 and TNFR2, but although oligomerization of soluble TNF does not enhance

TNFR1 signaling, TNFR2 activation has been shown to be highly dependent on TNF oligomerization (23, 32). Furthermore, soluble CD40L, which benefits only moderately from ligand oligomerization, has a relatively poor affinity of 7.1 nM, whereas EDA-A1, which interacts with EDAR with an affinity of 0.05 nM, still gains activity upon oligomerization (33). Indeed, we recently addressed the relevance of ligand oligomerization for affinity by help of GpL fusion proteins for soluble TWEAK, which poorly stimulate Fn14-mediated induction of the classical NF κ B target IL8, and for soluble CD95L, which fails to trigger robust apoptosis induction (9, 10). In these two cases, we noticed no major effect of ligand oligomerization on receptor occupancy and apparent affinity. Noteworthy, phylogenetic

TNFSF Ligand-TNFRSF Receptor Interactions

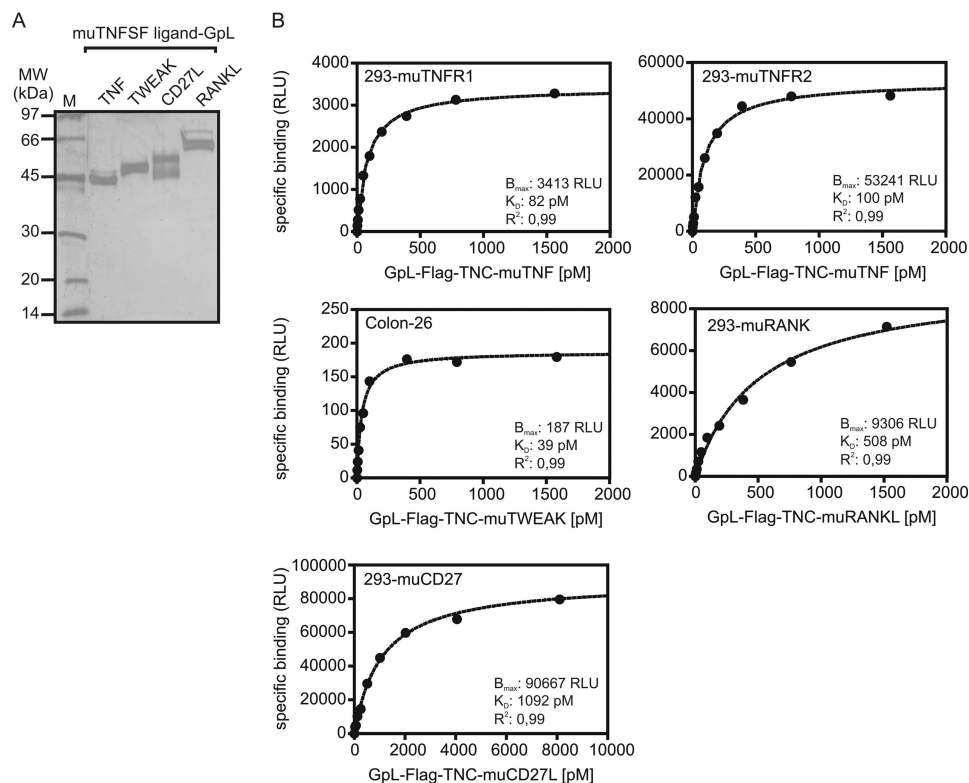


FIGURE 6. Saturation binding studies of murine TNFSF ligand-TNFRSF receptor interactions. *A*, 100 ng of the indicated anti-FLAG M2-agarose affinity-purified murine GpL-TNFSF ligands were separated by SDS-PAGE and visualized by silver staining. *B*, HEK293 transiently transfected with expression plasmids encoding the indicated murine TNFRSF receptors along with mock-transfected HEK293 cells were cultivated overnight in 24-well plates (half-plate control cells and half-plate receptor transfectants). The next day, cells were pairwise-incubated with increasing concentrations of the indicated GpL-TNFSF ligand fusion proteins for an additional hour at 37 °C. After removal of unbound molecules, the nonspecific binding values (= mock transfectants) were subtracted from the total binding values (receptor transfectants) to obtain specific binding values. To analyze the murine TWEAK-Fn14 interaction, Colon-26 cells were cultivated in 24-well plates. Half of the wells were preincubated with an excess (2 μ g/ml) of FLAG-TWEAK to block Fn14 binding. Cells were then pairwise incubated with GpL-FLAG-TNC-muTWEAK for an additional hour at 37 °C, and after removal of unbound molecules, specific binding was again calculated as the difference of total binding (untreated cells) and nonspecific binding (blocked cells). Specific binding values were finally fitted by non-linear regression to a single binding site interaction plot with the GraphPad Prism5 software. One representative experiment is shown for each of the murine TNFSF ligand-TNFRSF receptor interactions. For the number of experiments and statistics of each interaction, see Table 4.

TABLE 4

Murine TNFSF ligand-TNFRSF receptor affinities derived from cellular binding studies at 37 °C

All binding studies considered were performed at 37 °C and showed R^2 values for non-linear regression of specific binding data >0.95.

Interaction	Sequence identity in ectodomain (%)		K_D^a (nM)		No. of experiments ^a	
	Ligand	Receptor	Mouse	Human	Mouse	Human
TNF-TNFR1 (TNFSF2-TNFRSF1A)	76	71	0.086 \pm 0.008	0.040 \pm 0.010	4	5
TNF-TNFR2 (TNFSF2-TNFRSF1B)	76	57	0.089 \pm 0.012	0.082 \pm 0.028	4	4
CD27L-CD27 (TNFSF7-TNFRSF7)	63	61	1.186 \pm 0.111	0.801 \pm 0.14	3	4
RANKL-RANK (TNFSF11-TNFRSF11A)	85	78	0.444 \pm 0.118	0.337 \pm 0.048	4	8
TWEAK-Fn14 (TNFSF12-TNFRSF12A)	89	78	0.051 \pm 0.015	0.152 \pm 0.05	3	3

^a Data of the human interactions are from Table 3.

subgroups of the TNFRSF such as TNFR1 and TNFR2, the various TRAIL receptors, or TACI, BCMA, and BAFFR have quite similar affinities but nevertheless can differ in their ligand oligomerization requirement for activation. This is evident from the already mentioned TNFR1-TNFR2 system but also from the TACI-BCMA-BAFFR group. Here, as discussed above, TACI and BCMA activation highly benefits from oligomerization of its ligands APRIL and BAFF, although this plays no role in BAFFR stimulation by BAFF (21, 34).

There is increasing evidence in the literature that the superior response of the TNFRSF receptors discussed above to oligomerized or membrane-anchored soluble ligand trimers is due to the secondary clustering of the initially formed TNFSF

ligand₃-TNFRSF receptor₃ complexes. In the supramolecular TNFSF ligand-TNFRSF receptor clusters, certain pathways may then be more robustly activated by trans-activation of signaling proteins associated with the primary TNFSF ligand₃-TNFRSF receptor₃ complexes (8). The molecular mechanisms that drive/assist this secondary interaction are poorly understood but may involve receptor-receptor interaction via the PLAD and/or topological factors (spatial pre-orientation of membrane-bound molecules, association with the cytoskeleton, limited diffusibility). Thus, the K_D values of soluble TNFSF ligands for cell-expressed TNFRSF receptors, as determined in this study, alone are not in any case sufficient to allow modeling of receptor activity. Nevertheless, these values are also an

essential prerequisite for *in silico* analysis of activation of TNFRSF receptors superiorly stimulated by membrane-bound ligands. First, the K_D values of the soluble ligand molecules could be used to initially calculate binding of membrane TNFSF ligand to cell-expressed TNFRSF receptors by considering the membrane-localized molecules as soluble molecules in a spherical shell of the thickness of 1–2 receptor molecules. Second, also TNFSF ligand molecules that primarily act in their membrane-bound form are often shed. Now, the resulting soluble trimers may activate specific signaling pathways that do not need secondary clustering of TNFSF ligand₃-TNFRSF receptor₃ complexes (there is evidence for this possibility for TWEAK and CD95L (8)) and for such cases the K_D values determined in our study would be the relevant parameter to describe receptor activation. Moreover, as has been demonstrated for CD95L (35), soluble TNFSF ligands may act as inhibitors of their corresponding membrane-bound form. In these cases the affinity of the soluble molecule would again be a functional relevant parameter. It is worth mentioning that binding studies with GpL-TNFSF ligand fusion proteins also easily allow the determination of association and dissociation rate constants and the mean lifetime of ligand-receptor complexes (9, 10). The systematic evaluation of these parameters may give new insights into the question of how the dynamics/stability of the ligand-receptor complex contributes to the quality and quantity by which a certain TNFRSF receptor type activates intracellular signaling pathways. The simplicity of cellular binding studies with GpL-TNFSF ligand fusion proteins obviously has considerable potential for the evaluation and screening of substances interfering with the TNFSF ligand-TNFRSF receptor interactions.

Affinities of the huge majority of the known TNFSF ligand-TNFRSF interactions can be found in the scientific literature. However, the affinities available for a distinct TNFSF ligand-TNFRSF interaction often differ by several orders of magnitude. This inconsistency may not only limit *in silico* analysis of TNFSF ligand-TNFRSF receptor systems comprising several ligands and receptors under non-saturating conditions but might also affect conclusions that are based on pharmacological data. The major issue that could explain this inconsistency is the fact that in the various binding studies receptor molecules with a different number of protomers have been considered. In most studies using cell-free methods recombinant soluble TNFRSF receptor fusion proteins containing 1 or 2 receptor protomers have been used. Crystallographic studies, however, show that a TNFSF ligand trimer typically interacts with three receptor molecules (8).

In the absence of ligand, receptor dimers/trimers have been observed for several members of the TNFRSF, including TNFR1, TNFR2, CD40, CD95, and the TRAIL death receptors that are formed due to the autoaffinity of the PLAD. The autoaffinity of the PLAD of TNFRSF receptors is however rather low. This is evident from the fact that recombinant ectodomains of TNFRSF receptors typically form monomers. Indeed, even the PLAD of TNFR1, a TNFRSF receptor with high autoaggregating activity, is below 1 μM (36). We therefore assume that the majority of cell-expressed TNFRSF receptors occur in the form of monomers with low ligand affinity, which are in equilibrium with a minor species of dimeric/trimeric receptors with higher avidity and thus higher apparent affinity. As the dimeric/trim-

eric receptor species preferentially interacts with the trimeric TNFSF ligand molecules under formation of much more stable ligand-receptor complexes, the ligand-free dimeric/trimeric receptor species are continuously removed from the equilibrium with their monomers until the equilibrium between trimeric ligand and dimeric/trimeric receptor species has been reached. As a consequence, although initially only a few receptor molecules might bind their ligand with avidity and high affinity, over time a significant fraction of TNFRSF receptors becomes liganded despite low ligand concentrations, and this is what we have measured in our equilibrium binding studies. Thus, the low affinities determined in cell-free assays with monomeric recombinant receptor variants presumably correspond to the ligand affinity of cell-expressed monomeric TNFRSF receptors that typically may barely contribute to total ligand binding. In the case of TNFRSF receptor types with a rather small difference in ligand affinity between a single receptor protomer and receptor dimer/trimers, the low affinity binding of trimeric TNFSF ligands to monomeric receptors might significantly contribute to ligand binding especially when the receptor type has a poor autoaffinity.

A second issue that might contribute to the higher affinities that are typically found in cellular binding studies is the fact that the formation and stability of TNFSF ligand-TNFRSF complexes in intact cells can be supported by auxiliary processes, such as *e.g.* interaction with the cytoskeleton or lipid rafts.

We overcame the limitations of cell free-binding studies by performing cellular binding assays at 37 °C and ascertained a comprehensive set of apparent affinities for most TNFSF ligand-TNFRSF receptor interactions. We have used the same method of TNFSF ligand labeling, namely genetic fusion with the luciferase of *G. princeps* that not only improved the comparability of the data obtained for different interactions but also ensured high reproducibility from batch to batch. It is also worth mentioning that the genetic fusion of the GpL domain results in only one defined molecular species, whereas the majority of biochemical labeling methods, *e.g.* with iodine-131 or fluorochromes, results in a mixture of molecular species with a different degree of label load and unknown activity. In contrast to the use of such biochemically labeled ligands, the use of the GpL domain in our studies ensures that the activities measured in functional assays are indeed a property of the molecular species that is responsible for receptor occupation in corresponding binding studies. In sum, we think that our systematic study offers the scientific community the currently most comprehensive and best suited panel of affinities of TNFSF ligand-TNFRSF receptor interactions for *in silico* studies of ligand-receptor systems of the TNF family.

Author Contributions—I. L. performed binding studies and functional studies, analyzed data, and wrote the manuscript. S. F., A. W., A. F., J. T., and J. A. C. A. performed binding studies and functional studies and analyzed data. V. S. and D. W. cloned and produced the various recombinant proteins used in the study. H. W. designed the project, analyzed data, and wrote the manuscript.

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