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Trimer Stabilization, Oligomerization, and Antibody-Mediated Cell Surface Immobilization Improve the Activity of Soluble Trimers of CD27L, CD40L, 41BBL, and Glucocorticoid-Induced TNF Receptor Ligand

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For many ligands of the TNF family, trimer stability and oligomerization status are crucial determinants of receptor activation. However, for the immunostimulatory ligands CD27L, CD40L, 41BBL, and glucocorticoid-induced TNF receptor ligand (GITRL), detailed information regarding these requirements is lacking. Here, we comprehensively evaluated the effect of trimer stability and oligomerization on receptor activation by these ligands. Treatment with soluble Flag-tagged CD27L, 41BBL, and GITRL minimally activated receptor signaling, while Flag-CD40L was highly active. Oligomerization with anti-Flag Abs further enhanced the specific activity of Flag-CD40L. 10-fold and of Flag-41BBL more than 200-fold, but it failed to activate Flag-CD27L and Flag-GITRL. We next investigated the relevance of trimer stability by introducing the tenascin-C (TNC) trimerization domain, yielding stabilized Flag-TNC-ligand trimers. Oligomerization with anti-Flag Abs potently activated signaling by Flag-TNC-CD27L and Flag-TNC-GITRL and, albeit to a lesser extent, Flag-TNC-CD40L and Flag-TNC-41BBL. Forced hexamerization, by introducing an Ig Fc domain, revealed that hexameric derivatives of Flag-TNC-41BBL, Flag-CD40L, and Flag-TNC-GITRL all activate receptor signaling with high efficiency, whereas hexameric Flag-CD27L variant left inactive. Finally, we attempted to selectively activate receptor signaling on targeted cells, by using Ab fragment (single-chain fragment variable region, scFv)-ligand fusion proteins, an approach previously applied to other TNF ligands. Target cell surface Ag-selective activation was achieved for scFv-41BBL, scFv-CD40L, and scFv-GITRL, although the latter two displayed already significant activity toward Ag-negative cells. In conclusion, our data establish that trimeric CD40L is active, 41BBL requires hexamerization, GITRL requires trimer stabilization, and CD27L requires trimer stabilization and oligomerization. Furthermore, surface immobilization might be exploited to gain locally enhanced ligand activity. The Journal of Immunology, 2009, 183: 1851–1861.

ligands of the TNF family are crucially involved in the maintenance of immune homeostasis, for example, by providing death signals to superfluous immune cells or, alternatively, by providing costimulatory immune signals. Important examples of the latter are CD27L, 41BBL, and glucocorticoid-induced TNF receptor ligand (GITRL), controlling various facets of the T cell response, and CD40L, providing a pivotal costimulatory signal to APCs, such as dendritic cells (DCs) and B cells (1–5). In accordance with these regulatory functions, the aforementioned ligands have been implicated in the development of autoimmune disease, but they also represent attractive targets for cancer immunotherapy. The aim in cancer immunotherapy is to exploit the immune stimulatory capacity of these molecules to boost the anti-tumor immune response. To date, attention has mainly focused on receptor-specific Abs. However, the use of agonistic Abs might be adversely affected by some of the intrinsic properties of Abs. For example, Abs can elicit Ab-dependent cellular cytotoxicity, an obviously deleterious side effect when aiming for costimulation. Therefore, ligand-based therapeutic approaches may in certain cases be better suited to achieve the intended effect than are agonistic Abs. However, to rationally design ligand-based therapeutics, knowledge on the requirements for ligand-mediated activation of the corresponding TNF receptors is of critical importance.

The obligate structural prerequisite for receptor binding is the phylogenetically conserved carboxyl-terminal TNF homology domain (THD) present in all TNF family members (6, 7). The THD is further responsible for ligand trimerization. Most ligands of the TNF family are type II transmembrane proteins. However, most of these ligands can be also found in a soluble form in vivo, due to proteolytic processing or alternative splicing (6, 7). Additionally, recombinant soluble variants have been generated experimentally by genetic engineering. Importantly, the ability of transmembrane vs soluble ligand variants to activate their corresponding receptor(s) may vary. In this regard, three distinct patterns of activation of TNF receptor family members have been reported.

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In the first instance, the receptor is as responsive to activation by the transmembrane ligand as it is responsive to activation by the corresponding soluble trimeric ligand. An example is TNFR1, which is efficiently activated by membrane TNF and by soluble TNF in the picomolar range (8). Further oligomerization of soluble ligand trimers, for example, via Ab-mediated crosslinking, has no additional effect on receptor activation in these cases (9).

In the second instance, TNF receptors efficiently bind the transmembrane as well as the soluble form of a ligand, but they fail to become robustly activated by the latter. Examples are the interaction of human CD95L with CD95, APRIL with TACI, OX40L with OX40, human TRAIL with TRAILR2, and TNF with TNFR2 (8–12). However, in this case secondary oligomerization of soluble ligand trimers converts these molecules into highly active agonists. Typically, the specific activity is increased by several orders of magnitude. One example is oligomerization of a Flag-tagged variant of soluble CD95L, with anti-Flag Ab M2, which increases the specific activity up to a factor of 1000 (9). Importantly, a genetically engineered hexameric CD95L fusion protein was found to be comparably active as anti-Flag Ab oligomerized Flag-CD95L trimers (13), indicating that hexamerization of CD95L is sufficient to fully activate the receptor CD95. An alternative mechanism that allows efficient TNF receptor activation by inactive/poorly active ligand trimers is immobilization. For instance, binding of soluble CD95L or soluble APRIL to components of the extracellular matrix converts these ligands into highly active compounds (14–16).

In the third instance, a soluble TNF ligand, despite forming trimers, fails to interact with its corresponding receptor and consequently fails to activate receptor signaling. An example is a murine CD95L variant solely comprising the THD. Binding of this ligand variant to murine CD95 can be restored by stabilizing the trimeric organization of the molecule. Note that although trimer stabilization of murine CD95L restores receptor binding, it is still not sufficient to properly activate its receptor. The latter still requires secondary oligomerization of ligand trimers (17).

The characteristics of inactive/poorly active soluble ligand trimers may be exploited therapeutically by targeted immobilization. Targeted immobilization of a TNF ligand can be achieved by genetic fusion to a single-chain fragment variable region (scFv) Ab fragment. Ab fragment-mediated binding of a scFv-ligand fusion protein to its target, for example, a stromal protein or a tumor-associated target Ag, results in local immobilization and activation of the ligand domain. In the case of CD95L, Ab fragment-mediated binding to a targeted stromal component or a cell surface Ag has been shown to yield up to a 1000-fold increase in the efficiency for triggering CD95-induced cell death (18–20). Importantly, scFv-CD95L fusion proteins are selectively activated on targeted cells or tissues only and are inactive on target Ag-negative cells and tissues. Thus, targeted immobilization may be an effective way to reduce or avoid side effects on target Ag-negative cells and tissues (21).

The obvious relevance of oligomerization status, trimer stability, and surface immobilization for ligand activity prompted us to determine the importance of these cues for activation of CD27, CD40, 41BB, and GITR by their corresponding ligands. We demonstrate that the activity of soluble trimeric variants of CD27L, CD40L, and 41BBL can be strongly increased by oligomerization or cell surface immobilization, whereas a stabilized trimeric GITR variant is already highly active per se. These findings are of principal relevance for the experimental and biotechnological use of recombinant variants of the aforementioned ligands. Additionally, these findings may also aid in unraveling the biological and physiological consequences of the shedding of these transmembrane proteins.

Materials and Methods

Expression vectors encoding the various Flag-tagged TNF ligand variants are derived from a variant of pCR3 (Invitrogen), which was provided by P. Schneider (University of Lausanne, Epalinges, Switzerland) and encodes an Ig signal peptide followed by a Flag tag. To obtain the various “Flag” variants, ampiclons of 41BBL, CD27L, CD40L, and GITR-L encoding the amino acids indicated in Fig. 1A including a stop codon were inserted in frame 5′ to the Flag tag by standard cloning techniques. The Flag-tenascin-C (TNC) fusion proteins were obtained similarly by insertion of the ampiclons in a modified version of the plasmid were the Flag tag has been replaced by a Flag-TNC domain. The Fc-Flag fusion protein encoding constructs were based on the pCR3-derived plasmid PS1158 (gift of P. Schneider, University of Lausanne, Epalinges, Switzerland), which encodes an N-terminal HA signal peptide followed by the Fc domain, a linker, and the THD of Fasl. To obtain the various Fc-Flag fusion proteins, the FasL part of PS1158 was removed and replaced by Flag-ligand or Flag-TNC-ligand fragments derived from the aforementioned constructs. The sc40 fusion proteins were obtained by replacing the leader encoding fragment of the Flag-ligand encoding plasmid by an ampiclon encoding the leader sequence plus the sc40 cDNA.

Production and purification of recombinant proteins

The different ligand fusion proteins were produced in HEK293 cells by transient transfection of the corresponding expression vectors. In brief, cells were electroporated (50 × 10⁶ cells/ml, 4-mm cuvette, 250 V, 1800 μF, maximum resistance) with 30 μg of DNA in 1 ml of culture medium containing 10% FCS using an EasyJet Plus electroporator (PeqLab). Electroporated cells were recovered overnight in RPMI 1640 with 10% FCS (PAA Laboratories), and the next day the medium was replaced by low serum RPMI 1640 (0.5% FCS). After 3–4 days supernatants were collected and purified by centrifugation. With the exception of Flag-tagged GITR-L, which was only poorly produced, the various Flag-tagged ligands were purified by affinity chromatography on anti-Flag M2 agarose beads (Sigma-Aldrich) and elution with TBS containing 100 μg/ml Flag peptide (Sigma-Aldrich). The fractions containing the recombinant proteins were finally dialyzed against PBS and stored at −20°C for further analysis.

Gel filtration chromatography

Purified ligands (50 μl, 50–300 μg/ml) were analyzed by size exclusion chromatography on a BioSep-SEC-S3000 (300 × 7.8) gel filtration column (Phenomenex) equilibrated with PBS at a flow rate of 1 ml/min. Calibration of the column was conducted with the column performance check standard aqueous SEC 1 solution (Phenomenex) containing bovine thyroglobulin (670 kDa), IgG (150 kDa), OVA (44 kDa), and myoglobin (17 kDa).

FACS analysis

To determine cell surface expression of 41BB, CD27, CD40, and GITR, cells were incubated with ice-cold PBS, cell-associated immunofluorescence was determined by FACS analysis. To determine ligand binding to the corresponding cell surface receptors, cells were washed with PBS and incubated on ice for 30 min with the various ligand variants together with 1 μg/ml monoclonal anti-Flag mAb M2 (Sigma-Aldrich) and PE-labeled, mouse IgG-specific goat Abs (1 μg/ml Sigma-Aldrich). After three washes with ice-cold PBS, samples were analyzed using a FACS-Calibur (BD Biosciences).

SDS-PAGE and Western blot analysis

Samples were boiled (95°C, 5 min) in reducing (with 25 mM DTT) and non-reducing (so DTT) Laemmli sample buffer (0.5% SDS, 2.5% glycerol, 15 mM Tris (pH 8.0)) and were separated by SDS-PAGE. Protein gels were either visualized by silver staining or Coomassie blue (PageBlue protein staining solution; Fermentas) staining or were transferred to nitrocellulose membranes for analyzing by Western blot. In the latter case membranes were incubated with PBS containing 0.05% Tween 20 and 5% dry milk to block unspecific binding. And Flag-tagged proteins were detected with anti-Flag mAb M2 (0.5 μg/ml; Sigma-Aldrich), HRP-coupled secondary Ab (Dako), and the Amer sham ECL. Western blotting detection reagents (GE Healthcare). To prepare samples for Western blot detection of phosphorylated IκBα (anti-pIκBα; Cell
Signaling Technology) and total IgBe (anti-IgBe; Santa Cruz Biotechnology), cells were harvested into ice-cold PBS, washed twice with PBS, and were then directly lysed in 4× Laemmli sample buffer (8% SDS, 0.1 M DTT, 40% glycerol, 0.2 M Tris (pH 8.0)) supplemented with phosphatase inhibitor cocktails I and II (Sigma-Aldrich). After sonification (10 s) cell lysates were boiled and further processed as described above.

FIGURE 1. Subunit structure of soluble variants of 41BBL, CD27L, CD40L, and GITRL. A, Domain architecture of the Flag, Flag-TNC, and Fc-Flag fusion proteins of the soluble TNF ligands used in this study. Fc refers to Fc fragment of human Ig G1, and Flag indicates the presence of the Flag epitope D-Y-K-D-D-D-K. TNC represents aa 110–139 of chicken TNC that adopts a disulfide bonded trimeric structure. B and C, The indicated ligand variants were transiently produced in HEK293 cells and purified by affinity chromatography on anti-Flag agarose. Purified proteins were treated with reducing and nonreducing sample buffer, separated by SDS-PAGE, and either directly visualized by Coomassie staining (B) or after transfer to nitrocellulose detected by Western blot analysis with anti-Flag mAb M2 (C). D, The indicated purified ligand variants were subjected to gel filtration on a BioSep-Sec-S3000 column, and molecular masses were calculated based on the elution volume of the standards thyroglobulin (670 kDa), IgG (150 kDa), OVA (44 kDa), and myoglobin (17 kDa).
**ELISA analysis**

Cells were seeded (2 × 10^6 cells/well) in 96-well tissue cultures plates in 100 μl of RPMI 1640 medium with 10% FCS and grown overnight. On the next day, medium was exchanged to minimize the background of constitutive IL-8 production, and cells were stimulated for 6 h with the indicated concentrations of the various ligand proteins. Oligomerized ligand samples were generated by incubation with anti-Flag mAb M2 (1 μg/ml; Sigma-Aldrich) for 30 min before cell stimulation. Supernatants were analyzed for production of IL-8 using the BD OptiEA Human IL-8 ELISA Set (BD Biosciences) according to the manufacturer's instructions.

**Isolation, cultivation, and stimulation of primary cells**

PBMCs were isolated from blood from healthy donors by density gradient centrifugation with lymphocyte separation medium (PAA Laboratories). T cells and monocytes were separated from PBMCs by MACS magnetic bead separation with anti-CD3- and anti-CD14-coated beads (Miltenyi Biotec), respectively. T cell proliferation was quantified with the Vybrant CFDA-SE (carboxyfluorescein diacetate succinimidyl ester) cell tracer kit (Invitrogen). Cells were incubated with 10 μM CFDA-SE for 15 min and washed three times with RPMI 1640 complemented with 10% FCS (PAA Laboratories). Cells were taken up in RPMI 1640 with 100 U/ml penicillin, 100 μg/ml streptomycin (PAA Laboratories), and 10% human serum supplemented with 5 μg/ml PHA (Sigma) and 20 U/ml IL-2 (Proleukin; Chiron). Cells were challenged every second day as indicated with CD27L-Flag-TNC (50 ng/ml) crosslinked with M2 Ab (1 μg/ml; Sigma-Aldrich) or without crosslinking, and every 4 days they received an additional 20 U/ml IL-2. After 9 days CFDA-SE fluorescence was measured with the FACS-Calibur (BD Biosciences).

Monocytes were seeded in 6-well plates (3 × 10^6 cells) with RPMI 1640, 10% FCS, and penicillin/streptomycin. For DC differentiation 100 ng/ml GM-CSF and 20 ng/ml IL-4 (ImmunoTools) were added every second day. On day 7 cells were stimulated as indicated with TNF (20 ng/ml), IL-1640, 10% FCS, and penicillin/streptomycin. For DC differentiation 100 ng/ml GM-CSF and 20 ng/ml IL-4 (ImmunoTools) were added every second day. On day 7 cells were stimulated as indicated with TNF (20 ng/ml), IL-1

**Results**

**Production and biochemical characterization of soluble CD27L, CD40L, 41BBL, and GITRL variants**

To comprehensively study the influence of trimer stability and oligomerization status on ligand activity, we generated three different formats of CD27L, CD40L, 41BBL, and GITRL (Fig. 1A). First, a variant of the respective soluble ligand consisting of the THD was fused with an amino-terminal Flag tag, yielding Flag-ligand variants. Second, the chicken TNC trimerization domain (22) was infused with an amino-terminal Flag tag, yielding Flag-ligand variants. The Flag tag present in all of the constructs served to allow affinity purification as well as experimental Ab-mediated secondary modifications, such as glycosylation. Under nonreducing SDS-PAGE conditions, all ligands with a TNC-Flag domain were reasonably expressed, reaching concentrations between 0.5 and 3 μg/ml PHA (Sigma) and 20 U/ml IL-2 (Proleukin; Chiron). Cells were challenged every second day as indicated with CD27L-Flag-TNC (50 ng/ml) crosslinked with M2 Ab (1 μg/ml; Sigma-Aldrich) or without crosslinking, and every 4 days they received an additional 20 U/ml IL-2. After 9 days CFDA-SE fluorescence was measured with the FACS-Calibur (BD Biosciences).

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**Binding of 41BBL-, CD27L-, CD40L-, and GITRL-fusion proteins to their corresponding cell surface-expressed receptors.** HT1080 cells were stably transfected with expression vectors encoding human 41BB, human CD27, human CD40, or human GITR. The corresponding transfectants (HT1080-41BB, HT1080-CD27, HT1080-CD40, and HT1080-GITR) together with the parental HT1080 cells were analyzed by FACS with PE-labeled anti-41BB, anti-CD27, anti-CD40, or anti-GITR and the corresponding isotype control Abs. To analyze binding of the various 41BBL-, CD27L-, CD40L-, and GITRL-fusion proteins to their cognate receptors, the corresponding transfectants and parental HT1080 cells were incubated on ice with ligand (1 μg/ml). Cell-bound ligands were then visualized by FACS, exploiting their Flag tag and complexes of anti-Flag mAb M2 and PE-labeled goat anti-mouse IgG.

**Analysis revealed that the majority of Flag-tagged 41BBL, CD27L, and CD40L eluted in peaks corresponding to trimers. Additionally, formation of high-molecular mass (weight) (HMW) aggregates was detected especially for Flag-CD40L and Flag-CD27L (Fig. 1D; for functional analysis, see below). The Flag-TNC-ligands eluted in a dominant peak corresponding to the size of trimers (Fig. 1D). However, a shoulder in the peaks indicated that also hexamers were formed. In contrast to their corresponding Flag-tagged variants, the Flag-TNC-ligands showed no or only very modest formation of HMW aggregates (Fig. 1D). Thus, the TNC domain improved/facilitated self-assembly of the soluble TNF ligands. Gel filtration analysis of the Fc-Flag-ligands further revealed that Fc-Flag-CD40L and Fc-Flag-41BBL eluted as a symmetric peak of ~400 kDa, indicative for a homogeneous molecule species (Fig. 1D), although a reliable estimation of molecular mass was not possible due to the poor resolution of the gel filtration column at higher molecular masses. Fc-Flag-41BBL in addition eluted as a peak of ~95 kDa, which corresponds in size to dimeric molecules. In contrast, Fc-Flag-CD27L migrated in a broad elution pattern, indicative of the formation of oligomers with a nondefined number of monomers. Fc-Flag-GITRL eluted in a major peak of 99 kDa and in a minor peak of 286 kDa. The major 99 kDa peak corresponded to the size of an Fc-Flag-GITRL dimer. This suggests that
the intrinsic trimerization capability of the isolated THD of GITRL is rather low.

**Binding of recombinant variants of CD27L, CD40L, 41BBL, and GITRL to their corresponding cell surface-expressed receptors**

Before assessing the receptor-activating potential, we verified whether the various ligand variants interact appropriately with their cognate receptors. Incubation of stable HT1080 transfectants expressing CD27, CD40, 41BB, or GITR, with the corresponding ligands revealed that all variants of CD40L, 41BBL, and GITRL selectively bound their corresponding receptors (Fig. 2). In contrast, no binding was observed on parental HT1080 cells (Fig. 2). Of the CD27L constructs, only the stabilized trimeric TNC-Flag-CD27L variant robustly bound to HT1080-CD27 cells (Fig. 2), whereas no specific binding was detected for Flag-CD27L and Fc-Flag-CD27L. Taken together, these data indicate that spatial fixation of the free amino terminus of soluble CD27L by TNC is necessary to stabilize the molecule and to facilitate receptor binding.

**Effect of oligomerization on the specific activity of CD27L, CD40L, 41BBL, and GITRL**

To delineate the receptor-activating potential of the various ligand trimers, stabilized trimers, and hexamers, the HT1080 transfectants expressing CD27, CD40, 41BB, and GITR were stimulated with anti-Flag agarose affinity-purified preparations of the different ligand variants, and IL-8 production was determined by ELISA (Fig. 3, A–D). All CD40L variants as well as Flag-TNC-GITRL and Fc-Flag-GITRL strongly induced IL-8 production in HT1080-CD40 and HT1080-GITR transfectants, starting at concentrations between 0.1 and 1 ng/ml and reaching maximum induction at 10–50 ng/ml (Fig. 3, C and D). Further oligomerization of these constructs using anti-Flag mAb M2 had only a moderate effect, which was most pronounced for Flag-CD40L yielding a shift in the
ED$_{50}$ values of more than one order of magnitude. Contrary to CD40L and GITRL, all 41BBL and CD27L variants were virtually unable to induce IL-8 production in the absence of anti-Flag oligomerization (Fig. 3, A and B). However, secondary oligomerization tremendously enhanced the activity of the stably trimerized Flag-TNC-41BBL and Flag-TNC-CD27L variants (Fig. 3, A and B), yielding ED$_{50}$ values of ~100 ng/ml. This strongly contrasts with the minimal IL-8 induction, observable in the absence of oligomerization at concentrations of up to 2000 ng/ml (Fig. 3, A and B). After oligomerization, the Flag-tagged 41BBL variant also induced IL-8, albeit significantly less than the corresponding TNC-stabilized variant. The hexameric Fc-Flag-41BBL variant minimally induced IL-8 in the absence of crosslinking, which was also only modestly enhanced by oligomerization with anti-Flag. Unlike the Flag-TNC-CD27L variant, Flag-CD27L and Fc-Flag-CD27L were practically inactive even after crosslinking.

**FIGURE 4.** Functional analysis of gel filtration-purified, homogeneous molecule species of 41BBL, CD27L, CD40L, and GITRL. A. Affinity chromatography-purified preparations of the various 41BBL variants were separated by gel filtration. Fractions corresponding to the 49 kDa peak of Flag-41BBL (fraction no. 34, lane 1), the 78 kDa peak of Flag-TNC-41BBL (fraction no. 32, lane 2), and the 406 kDa peak of Fc-Flag-41BBL (fraction no. 28, lane 3) shown in Fig. 1 were analyzed by SDS-PAGE and silver staining. The molecular masses of the marker proteins (M labeled lanes) are indicated in kDa. B. The IL-8-inducing activity of the gel filtration-purified ligands were analyzed in the presence and absence of anti-Flag mAb M2 (0.5 µg/ml) by stimulation of HT1080-41BB cells and ELISA analysis of the corresponding supernatants. C. Gel filtration fractions (see Fig. 1) corresponding to the 49 kDa peak of Flag-CD27L (fraction no. 33, lane 1) and the 99 kDa peak of Flag-TNC-CD27L (fraction no. 32, lane 2) or containing molecules in size corresponding to a calculated hexamer of Fc-Flag-TNC-CD27L (fraction no. 28, lane 3) were analyzed by SDS-PAGE and silver staining. D, HT1080-CD27 cells were stimulated with the indicated concentrations of Flag-CD27L and Flag-TNC-CD27L derived from the fractions shown in C or with the undiluted HMW fraction (fraction no. 23) of Flag-CD27L in the presence and absence of anti-Flag mAb M2 (0.5 µg/ml). After 6 h, IL-8 production was determined by ELISA. E. Flag-TNC-GITRL derived from the 99 kDa peak (fraction no. 31, lane 1) and Fc-Flag-GITRL derived from the 99 kDa peak (fraction no. 31, lane 2) were analyzed by SDS-PAGE and silver staining. F. IL-8-inducing activity of the molecular species shown in E was determined with and without anti-Flag oligomerization by the help of HT1080-GITR cells and ELISA analysis. G. Flag-CD40L derived from the 55 kDa peak (fraction no. 34, lane 1) was analyzed by SDS-PAGE and silver staining. H, HT1080-CD40 cells were stimulated in triplicates with the indicated mixtures of trimeric (fraction no. 34) and aggregated (fraction no. 23) Flag-CD40L molecule species for 6 h, and IL-8 production was again determined by ELISA. I. Material from gel filtration corresponding to HMW fractions of Flag-CD27L (pure) and Flag-CD40L (pure) as well as Flag-CD40L from the 55 kDa peak (1 µg/ml) and Flag-TNC-CD27L from the 99 kDa peak (1 µg/ml) were tested for binding to HT1080-CD40 and HT1080-CD27 transfectants by FACS. J, HT1080-CD40 cells were challenged in triplicates for 6 h with the indicated mixtures of Flag-CD40L derived from the HMW fraction (fraction no. 23) and the trimer peak (fraction no. 34) in the absence and presence of anti-Flag mAb M2 (0.5 µg/ml). IL-8 concentration in supernatants was then again determined by ELISA.
The classical NF-κB pathway, which is known to be activated by all of the TNF receptors investigated in our study, is crucially involved in the regulation of the IL-8 gene. We therefore analyzed by all of the TNF receptors investigated in our study, is crucially phosphorylation of the inhibitor of IκBα (IκBα) protein. Flag-TNC-41BBL and Flag-TNC-CD27L failed to trigger phosphorylation of IκBα (Fig. 3E). In accordance with the requirement of these molecules for oligomerization to elicit a strong IL-8 response (see Fig. 3, A and B), phosphorylation of IκBα was easily detectable upon ligand crosslinking with the anti-Flag mAb M2 (Fig. 3E). Flag-TNC-CD40L and Flag-TNC-GITRL, which showed already efficient IL-8 induction in the nonoligomerized state, also stimulated IκBα phosphorylation without further oligomerization (Fig. 3E).

As is evident from Fig. 1B–D, the anti-Flag affinity-purified ligands, especially the Flag and Fc-Flag variants, are heterogeneous and assemble into different molecular species. To prove that our observations regarding the relevance of trimer stabilization and oligomerization for ligand activity are not influenced by having in some cases mixtures of different molecular species, we recapitulated our corresponding key findings by analysis of gel filtration fractions containing single molecular species. As shown in Fig. 4A, Flag-41BBL and Flag-TNC-41BBL derived from the corresponding trimer peaks as well as Fc-Flag-41BBL from the “400 kDa” peak are highly pure. Analysis of IL-8 production using the gel filtration-purified proteins confirmed our previous findings obtained with the corresponding molecularly heterogeneous preparations. Thus, the trimeric 41BBL variants are practically inactive, but they gained high activity after anti-Flag mAb-mediated oligomerization (Fig. 4B). Moreover, as observed before with the molecularly heterogeneous preparations, the maximum response achieved with Flag-TNC-41BBL was higher than those induced by Flag-41BBL (Fig. 4B), suggesting that the TNC-stabilized molecule possesses an intrinsically higher receptor activation capability. After removal of the aggregated molecule species/impurities by gel filtration, Flag-CD27L derived from the 49 kDa peak remained as inactive as the total preparation, irrespective of oligomerization with anti-Flag mAb (Fig. 4, C and D). Furthermore, none of the gel filtration fractions containing Fc-Flag-CD27L induced IL-8, ruling out that an active subspecies of Flag-CD27L molecules was inhibited by inactive material in the previously investigated total preparation (data not shown). As expected, Flag-TNC-CD27L derived from the trimer peak was active, but again only upon oligomerization (Fig. 4D). Flag-TNC-GITRL derived from the 99 kDa peak showed high activity, which was only very modestly enhanced by anti-Flag (Fig. 4F) and thus behaved similar to the corresponding total preparation only purified by affinity chromatography (Fig. 3D). Notably, the 99 kDa dimeric peak of Fc-Flag-GITRL was poorly active, but it gained significant activity, almost reaching those of Flag-TNC-GITRL trimers upon anti-Flag oligomerization (Fig. 4, E and F). In view of the high oligomerization-independent activity of trimeric Flag-TNC-GITRL, it appears therefore possible that in this special case oligomerization did not serve to “activate” poorly active ligand trimers by aggregation, but instead facilitated formation of active trimers within aggregates of dimeric Fc-Flag-GITRL species. An analysis of the 286 kDa peak of Fc-Flag-GITRL was not performed due to the limited availability of the corresponding molecular species. The HMW species of Flag-CD40L and Flag-CD27L, which represent a major fraction in the corresponding affinity-purified preparations, were completely (Flag-CD27L) or largely (Flag-CD40L) inactive (Fig. 4, D, G, and H). The specific activity of Flag-CD40L derived from the HMW peak was <1% of anti-Flag mAb oligomerized Flag-CD40L derived from the trimer peak (Fig. 4H). Furthermore, HMW aggregates of Flag-CD40L failed to bind CD40 in FACS analysis and HMW aggregates of Flag-CD27L showed only weak binding even when used without further dilution (Fig. 4I). Thus, the HMW fractions obviously contain misfolded inactive ligand aggregates and not bioactive oligomers. To prove whether HMW aggregates of Flag-CD40L interfere with the activity of the active trimeric molecule species, we mixed a suboptimal concentration of the latter, where the beneficial effect of oligomerization is visible, with up to a 25-fold excess of the HMW fraction. In 1:1 and 1:5 mixtures of trimeric and HMW Flag-CD40L, there were no evidence for any effect of the HMW molecule species (Fig. 4J). Although the concentration of the HMW fraction, necessary to achieve a 25-fold excess over the trimer fraction, alone already showed weak activity, there was a slight reduction in IL-8 production in the corresponding mixture (Fig. 4J). Thus, in extreme unfavorable proportions trimeric Flag-CD40L might become inhibited by its aggregated counterparts.

Taken together, CD40L and GITRL already display high oligomerization-independent activity; the latter, however, needs stabilization, for example, by the TNC domain. In contrast, CD27L...
and 41BBL require oligomerization and stabilization of the trimeric molecule to activate their cognate receptors.

**Stabilized CD40L and CD27L also provide appropriate costimulation to primary cells**

To delineate whether stabilized ligands also possessed activity toward primary human target cells, the activity of oligomerized Flag-TNC-CD40L and Flag-TNC-CD27L was determined in primary cells by assessing DC maturation (for Flag-TNC-CD40L) and T cell proliferation (for Flag-TNC-CD27L). Treatment of IL-4/GM-CSF-stimulated monocytes with Flag-TNC-CD40L induced efficient maturation into DCs, as evidenced by the strong appearance of the DC marker CD86 (Fig. 5A). Additionally, the marker CD83 was also induced, albeit to a lesser extent (Fig. 5A). The effect of Flag-TNC-CD27L on T cell costimulation was assessed on primary T cells activated with suboptimal concentrations of PHA. Proliferation was markedly enhanced in the presence of oligomerized Flag-TNC-CD27L compared with PHA treatment alone, with a 20% increase in proliferating cells (Fig. 5B). Thus, both Flag-TNC-CD40L and Flag-TNC-CD27L displayed the anticipated costimulatory effects on human primary target cells.

**Trimeric scFv fusion proteins gain high activity after cell surface Ag binding**

A foreseeable problem that might limit the clinical applicability of costimulatory TNF receptor activation is the occurrence of systemic side effects and potential induction of autoimmunity. We and others have previously described an approach for CD95L and TRAIL, using Ab fragment-ligand fusion proteins, which might overcome or minimize this problem. Typically, scFv-ligand fusion proteins of CD95L and TRAIL only gain high bioactivity after cell surface immobilization and are essentially inactive/poorly active toward nontargeted cells/tissues (12, 18–20). Therefore, we next investigated the potential of targeted immobilization of CD27L, CD40L, 41BBL, and GITRL. To this end, soluble variants of the various ligands were fused to the scFv Ab fragment sc40 that specifically recognizes the tumor stroma Ag fibroblast activation protein (FAP) (23, 24) (Fig. 6, A and B). Subsequent gel filtration analysis of the sc40-Flag-CD40L fusion protein revealed a dominant peak corresponding to trimers (Fig. 6C). Additionally, minor formation of hexamers and HMW aggregates was detected. The fusion protein sc40-Flag-GITRL eluted in a mixture of monomers and trimers or tetramers (Fig. 6C). The fusion protein sc40-Flag-41BBL eluted in one dominant peak, which corresponded in size to a dimmer, and the fusion protein sc40-Flag-CD27L eluted as a monomer or as HMW aggregates (Fig. 6C). Importantly, incubation of HT1080 transfectants expressing FAP revealed that all fusion proteins efficiently interact with cell surface-expressed FAP (Fig. 7A, right panel). Moreover, all fusion proteins with the exception of sc40-Flag-CD27L also interacted robustly with their corresponding receptor (Fig. 7A, left panel). Not surprisingly, based on the lack of CD27 binding, sc40-Flag-CD27L left inactive in the presence of both FAP-positive and FAP-negative HT1080 cells (Fig. 7B). Of the other fusion proteins capable of binding both FAP and their cognate receptor, 41BBL is of particular interest based on the fact that trimeric variants of this ligand (Flag-41BBL and Flag-TNC-41BBL) were inactive. Thus, 41BBL has ideal characteristics for target cell-selective activation of receptor signaling. As expected, in the presence of FAP-negative cells, sc40-Flag-41BBL did not induce IL-8 production. In contrast, sc40-mediated binding to FAP-positive cells resulted in high IL-8 production in cocultured HT1080-41BB cells (Fig. 7B).

In accordance with the fact that trimeric forms of CD40L were capable of activating CD40, the sc40-Flag-CD40L fusion protein already displayed strong activity in the absence of FAP expression (Fig. 7B). Nevertheless, sc40-mediated binding of

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**FIGURE 6.** Generation of single-chain fusion proteins of 41BBL, CD27L, CD40L, and GITRL recognizing the cell surface Ag FAP. A, Domain architecture of fusion proteins of 41BBL, CD27L, CD40L, and GITRL with the FAP-recognizing single-chain fragment sc40 (B and C). Purified sc40-Flag-41BBL, sc40-Flag-CD27L, sc40-Flag-CD40L, and sc40-Flag-GITRL were analyzed by SDS-PAGE under reducing and non-reducing conditions followed by Western blotting with anti-Flag mAb M2 (B) and by gel filtration (C).
the fusion protein to FAP still shifted the ED_{50} value of the construct to an ~25-fold lower concentration. The activity of sc40-Flag-GITRL was enhanced 10-fold upon FAP-specific binding (Fig. 7 B). However, even after sc40-mediated immobilization the activity of sc40-Flag-GITRL was still approximately two orders of magnitude lower than the activity of Flag-TNC-GITRL. The rather low specific activity of FAP-bound sc40-Flag-GITRL is in agreement with the negligible activity of anti-Flag mAb-oligomerized Flag-GITRL.

Simultaneous incorporation of the TNC and Fc domain markedly increases the activity of 41BBL

Our data show that the TNC domain primarily improves receptor binding of corresponding TNC ligand variants, whereas introduction of the Fc domain drives hexamer formation and can thereby overcome the requirement for secondary oligomerization. The rather low specific activity of FAP-bound sc40-Flag-GITRL is in agreement with the negligible activity of anti-Flag mAb-oligomerized Flag-GITRL.

Discussion

In this study, we comparatively analyzed the activity of oligomerized, stabilized, and immobilized variants of CD27L, CD40L, 41BBL, and GITRL. Oligomerization of the ligands was achieved by secondary crosslinking with the mAb M2 recognizing a Flag tag present in all proteins studied; this led to higher order aggregates of varying nondefined stoichiometry. Furthermore, genetic fusion with the dimerizing Fc domain of human Ig G1 resulted in thus with molecular masses between 350 and 500 kDa (Fig. 8, B and C). Importantly, both Fc-Flag-TNC-41BBL and Fc-Flag-TNC-GITRL specifically interacted with their cognate receptor (Fig. 8D) and were strikingly efficient in inducing IL-8 production in HT1080-41BB and HT1080-GITR transfectants (Fig. 8E). Taken together, our results indicate that combining the TNC trimerization domain and the Fc domain in one molecule yields highly active ligands.
RELEVANCE OF OLIGOMERIZATION FOR TNF LIGAND ACTIVITY

Figure 9. Inactive/poorly active TNF ligand trimers can be converted to highly active molecules by oligomerization or cell surface immobilization. A, Scheme of assembly of Flag-Tag-TNC, Flag-TNC, Flag-FC, and Flag-FC-TNC variants of TNF ligands. B and C, Poorly active Flag-tagged (or Flag-TNC-tagged) trimeric TNF ligand variants gain high activity after oligomerization by anti-Flag (B) or by genetic engineering (fusion with an FC or FC-TNC domain) leading to hexamer formation (C). Trimer-forming single-chain (scFv) fusion proteins of poorly active TNF ligands become highly active and mimic their corresponding transmembrane form upon binding to their cognate cell surface Ag (D).

The TNC trimerization domain enhanced to a varying extent (10-fold to >1000-fold decrease in ED50 values) the intrinsic activity of all TNF ligands studied herein, and similar effects have been demonstrated for TRAIL, OX40L, and CD95L elsewhere (11, 17). The TNC trimerization domain might serve therefore as a global means to increase the activity of recombinant TNF ligands, allowing much lower doses of proteins to be applied in therapy. A similar stabilization and enhancement of human GITRL has recently also been achieved by generating a leucine zipper fusion protein, shown to prevent trimer dissociation (27). In fact, inactivation of soluble GITRL by trimer dissociation seems to be of particular importance. The crystal structure of human GITRL revealed a smaller intersubunit interface than for all other TNF ligands, involving only the side chains of ~10 residues and an area of 1.2 Å (2) instead of the usual ~40 residues and 6 Å (2, 27). Accordingly, in gel filtration analysis nonstabilized GITRL eluted as a mixed population of monomers and trimers, whereas leucine zipper-stabilized GITRL eluted as a single molecular species corresponding to trimers. Note that murine GITRL is unique in forming dimers instead of trimers (30, 31). This might correspond to the finding that an FC fusion protein of murine GITRL displays high activity (32). Introduction of the TNC domain was not able to overcome the requirement of TNF ligands for oligomerization to gain high activity (Fig. 3). However, introduction of an FC domain, thereby forcing formation of ligand hexamers, yielded highly bioactive molecules, which were functionally almost equivalent to M2-oligomerized ligand trimers. Thus, FC fusion proteins of trimeric ligand variants, requiring oligomerization to display significant activity, have already high activity in the absence of crosslinking Abs, and further addition of the latter showed only a minor effect (Fig. 3, A and B). Fusion proteins with an amino-terminal bipartite FC-TNC domain seem to be therefore the format of choice for the construction of recombinant TNF ligands with constitutively high activity (Fig. 8).

Possible complications that can be expected during a clinical exploitation of immune activation strategies that target and ubiquitously activate the costimulatory power of the CD40L-CD40, CD27L-CD27, 41BBL-41BB, or GITR-L-GITR systems are systemic inflammatory side effects and autoimmunity (1–5). There-fore, we evaluated whether the above-reported characteristics of inactive/poorly active soluble ligand trimers might be exploited therapeutically by targeted immobilization. Targeted immobilization can be achieved by the genetic fusion of a soluble TNF ligand to an Ab fragment (scFv). Selective scFv-mediated immobilization
of such scFv-ligand fusion proteins to their cognate target Ag confers a high receptor activating potential to their ligand domains of normally inactive/poorly active soluble variants (11, 12, 18–20). Thus, Ag-bound scFv-ligand fusion proteins act as mimetics of the corresponding transmembrane TNF ligands. Provided the use of a restrictively expressed target Ag, the scFv fusion proteins activate TNF receptor signaling locally on Ag-expressing cells and tissues, as for instance demonstrated previously for CD95L, TRAIL, and OX40L (11, 12, 18–20). Based on our data, the most promising candidate for this approach of the four ligands investigated here is 41BBL, as it is inactive as a soluble trimer. Indeed, a fusion protein of 41BBL with sc40, an Ab fragment recognizing the cell surface Ag FAP, resulted in a >1000-fold increase in activity upon binding to FAP-positive cells, with no activation of receptor signaling in the absence of FAP. As expected from the significant activity of soluble CD40L trimers, the corresponding scFv fusion protein of CD40L (sc40-Flag-CD40L) was already highly active without FAP binding. Nevertheless, FAP-specific binding caused still a significant enhancement of receptor activation (Fig. 7B). Taken together, at least the immobilization-dependent increase in the activity of sc40-Flag-41BBL offers the chance of achieving locally antigen-directed activation of TRAIL-R2 by a soluble TRAIL derivative. Onco- gene 20: 4101–4106.


