Studies of Binding of Tumor Necrosis Factor (TNF)-like Weak Inducer of Apoptosis (TWEAK) to Fibroblast Growth Factor Inducible 14 (Fn14)*

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Background: TWEAK and its receptor Fn14 are targets in oncology and autoimmunity.

Results: Ligand oligomerization has no major effect on Fn14-TWEAK interaction but strongly enhances TWEAK-induced IL8 production.

Conclusion: Avidity is irrelevant for TWEAK trimer binding to Fn14 but required for robust IL8 induction.

Significance: Enhanced activity of oligomerized TWEAK trimers is not related to an avidity-related increase in Fn14 occupancy.

To perform highly sensitive cellular binding studies with TNF-like weak inducer of apoptosis (TWEAK), we developed a bioluminescent variant of soluble TWEAK (GpL-FLAG-TNC-TWEAK) by fusing it genetically to the C terminus of the luciferase of *Gaussia princeps* (GpL). Equilibrium binding studies on human (HT1080 and HT29) and murine (Renca and B16) cell lines at 37 °C revealed high affinities of human TWEAK from 53 to 112 pM. The dissociation rate constant of the TWEAK-Fn14 interaction was between 0.48 × 10^{-3} s^{-1} (HT29) and 0.58 × 10^{-3} s^{-1} (HT1080) for the human molecules, and the association rate constant obtained was 3.3 × 10^{6} M^{-1} s^{-1} for both cell lines. It has been shown previously that oligomerization of soluble TWEAK trimers results in enhanced Fn14-mediated activation of the classical NFκB pathway. Binding studies with GpL-FLAG-TNC-TWEAK trimers oligomerized by help of a FLAG tag-specific antibody gave no evidence for a major increase in Fn14 occupancy by oligomerized ligand despite strongly enhanced induction of the NFκB target IL8. Thus, aggregated complexes of soluble TWEAK and Fn14 have a higher intrinsic activity to stimulate the classical NFκB pathway and qualitatively differ from isolated trimeric TWEAK-Fn14 complexes. Furthermore, determination of IL8 induction as a function of occupied activated receptors revealed that the intrinsic capability of TNFR1 to stimulate the classical NFκB pathway and IL8 production was ~100-fold higher than Fn14. Thus, although ~25 activated TNFR1 trimers were sufficient to trigger half-maximal IL8 production, more than 2500 cell-bound oligomerized TWEAK trimers were required to elicit a similar response.

Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK)² is a typical member of the TNF ligand family and is thus expressed as a type 2 transmembrane protein (1). Membrane TWEAK consists of an N-terminal cytoplasmic domain followed by a single transmembrane domain that is separated by a stalk region from the C-terminal TNF homology domain (THD), which is characteristic for the TNF ligand family. Membrane TWEAK is efficiently processed in its stalk region by proteases of the furin family resulting in a soluble ligand essentially comprising the THD of the molecule. The THD is responsible for ligand trimerization and receptor binding, and soluble TWEAK is thus still able to interact with its receptor Fn14 and to trigger Fn14-associated signaling pathways (1).

Fn14 is an unusually small member of the TNF receptor family and the only unquestionably proved signal-transmitting receptor of TWEAK (1). It has also been reported that TWEAK binds to CD163 (2). There is evidence that this interaction interferes with Fn14 binding of TWEAK and results in internalization of CD163-TWEAK complexes by macrophages (3). CD163 binding of TWEAK thus seems to act as an antagonizing mechanism, but its relevance in vivo is currently unclear.

Expression of Fn14 is typically induced by growth factors and is accordingly particularly high after tissue damage. For example, induction of Fn14 has been reported in context of arthritis, ischemia, liver injury, intoxication of skeletal muscle, and glomerulonephritis (4–12). There is also often strong Fn14 expression in solid tumors (13). At the mRNA level, TWEAK expression has been demonstrated in a variety of cell lines and tissues (14). In contrast, detection of membrane TWEAK by FACS was so far only successful for IFNγ-stimulated monocytes, macrophages, dendritic cells, and a very few breast cancer cell lines (15–18). In view of the strong TWEAK processing activity of furin proteases, this points to an important role of soluble TWEAK, although there is also evidence that TWEAK mRNA is inefficiently translated.

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² The abbreviations used are: TWEAK, TNF-like weak inducer of apoptosis; NFκB, nuclear factor κB; THD, TNF homology domain; TNFR1, TNF receptor 1.
Stimulation of Fn14 results in the activation of signaling pathways that are also triggered by other members of the TNF receptor family. So stimulation with TWEAK results in strong activation of the alternative NFκB pathway, but often there is also activation of MAPKs, Akt, and the classical NFκB pathway (1). Although activation of the alternative NFκB pathway by TWEAK is typically strong in all cell lines, the extent of activation of other unquestionably proven pathways is quite variable and depends upon the cell type.

Although Fn14 contains no death domain and is thus not a bona fide death receptor, TWEAK induces necrotic and/or apoptotic cell death in a limited number of cell lines (14, 17, 19–21). Cell death induction has been attributed to the production of endogenous TNF and subsequent stimulation of the death receptor TNFR1 (17, 20, 22). However, there is also evidence for TWEAK-induced cell death by an endogenous TNF-independent yet unidentified mechanism (17, 21, 22). The Fn14-associated signaling pathways listed above are involved in the orchestration of proliferative, inflammatory, and angiogenic processes. For example, TWEAK and Fn14 trigger proliferation of mesenchymal progenitor cells (5, 7, 23) and inhibit differentiation of chondrocytes, osteoblasts, and myocytes (5, 7, 23–25). In view of the wound healing-associated functions of Fn14 and TWEAK, these molecules are attractive therapeutic targets for the treatment of autoimmune diseases and ischemia-related tissue damages (1). Because of the broad and strong avidity of Fn14 on tumor cells and the potentially protumoral acting activities of the TWEAK-Fn14 system, the latter is also considered as a promising target for cancer treatment (13).

Despite the huge clinical interest in the exogenous control of the TWEAK-Fn14 system, only a few quantitative data are available concerning the TWEAK-Fn14 interaction. Here, we describe the use of GpL-FLAG-TNC-TWEAK, a bioluminescent fusion protein of soluble TWEAK with the *Gausia princeps* luciferase (GpL), to analyze the TWEAK-Fn14 interaction with high accuracy and sensitivity on intact cells. We determined the kinetic parameters of TWEAK binding to cell surface-expressed Fn14 and demonstrated that the enhanced activity of oligomerized TWEAK trimers is not related to an avidity-related increase in Fn14 occupancy.

**EXPERIMENTAL PROCEDURES**

**Cell Line and Reagents**—All cell lines (Hek293, C2C12, HT1080, HT29, B16, and Renca) were cultured in RPMI 1640 medium (PAA, Pasching, Germany) supplemented with 10% fetal calf serum (PAA, Pasching, Germany) and 2 mm l-glutamine at 37 °C. The pCR3-derived expression vector encoding secretable FLAG-TWEAK (amino acids 106–249) has been described elsewhere (20). Using the flanking EcoRI (5’) and Xbal (3’) sites, the TWEAK domain-containing DNA fragment of FLAG-TWEAK-pCR3 was subcloned in pCR3 derivatives with an N-terminal GpL-FLAG epitope-TNC or a Leader-hlgG1(Fc)-FLAG epitope encoding cassette to allow expression of secretable GpL-FLAG-TNC-TWEAK and Fc-FLAG-TWEAK. TNC refers to the trimerization domain of chicken tenasin C, which serves to stabilize the trimeric assembly of ligands of the TNF family and thus reduces formation of inactive misfolded protein species (26, 27). The *G. princeps* luciferase (GpL)-encoding DNA fragment used was a synthetic product of the complete reading frame of the GpL gene with human codon usage. The expression constructs encoding FLAG-TNF and GpL-FLAG-TNC-TNF were correspondingly obtained by exchange of the TWEAK domain-encoding DNA fragment with a DNA fragment encoding soluble TNF. Primary antibodies specific for p100/p52 and the FLAG epitope were from Upstate Biotechnology (Schwalbach, Germany) and Sigma, respectively. The CD163-specific antibody was purchased from BD Biosciences, and recombinant soluble CD163 as well as the anti-TWEAK antibody used for Western blotting were from R&D Systems (Wiesbaden-Nordenstadt, Germany).

**Binding Studies**—For equilibrium binding studies cells were grown overnight (1–2 × 10⁵ cells/well) in tissue culture 24-well multiplates. To determine nonspecific binding, cells in half of the wells were pretreated with an antagonist of the GpL-ligand variant of interest for 30 min at 37 °C. Access to the TWEAK receptor Fn14 was blocked with a high concentration (1.5 μg/ml; 4.7 nm) of conventional Fc-FLAG-TWEAK, a dimerized variant of the trimeric FLAG-TWEAK molecule (28), and TNFR1 binding was blocked using the TNFR1-specific antibody H398 (10 μg/ml), which was a kind gift of Prof. Klaus Pfizenmaier and Prof. Peter Scheurich (University of Stuttgart). Cells were then incubated with increasing concentrations of the GpL-FLAG-TNC-TWEAK or GpL-FLAG-TNC-TNF for 2 h at 37 °C. To remove unbound ligand, cells were rapidly washed 10 times by pushing plates twice in each of five 2-liter tanks containing ice-cold PBS. Cells were scraped into 50 μl of medium (RPMI, 0.5% FCS, room temperature) with a rubber policeman and transferred to black 96-well plates. Cells of one well were counted using a hemocytometer for later calculation of the average receptor number per cell. To quantify GpL activity in the various samples, the enzymatic reaction was initiated by addition of 10 μl of luciferase working solution (*Gausia* luciferase assay kit, New England Biolabs GmbH, Frankfurt am Main, Germany), and luminescence (1 s per sample) was immediately measured using a Luc 2 luminometer (Anthos Labtech Instruments, Krefeld, Germany). Luminescence activity of defined amounts of purified GpL-FLAG-TNC-TWEAK and GpL-FLAG-TNC-TNF was also measured to allow conversion of relative light units into molecule numbers. The luminescent activity of GpL dropped significantly within a few minutes (29). To minimize the error related to this decay, enzymatic reactions were started and analyzed in packages of six samples. Based on regular measurements of GpL fusion protein standards of known concentration, the decay-related error was <5%. Specific binding was defined as the difference of the total binding values (samples without antagonist pretreatment) and corresponding nonspecific binding values (samples pretreated with antagonist).

To calculate the average number of TWEAK-binding sites per cell (*N*₃₂), we determined the luciferase activity of purified GpL-FLAG-TNC-TWEAK standards of known concentration to obtain the luciferase activity per GpL domain (*A*₃₂) three per TWEAK trimer). Together with the known number of cells per well (*N*₃₂) and the maximum specifically bound GpL activity (*P*₃₂) from the corresponding binding studies, it was then possible to calculate the average number of binding
sites per cell ($N_{BS} = B_{max} \times CN_{well}^{-1} \times A_{GpL-dom}^{-1}$). The latter are defined here as the number of GpL domains that specifically bind to cells. Thus, one binding site corresponds to a single receptor irrespective of the fact that a TWEAK trimer can interact with three receptor molecules.

For homologous competition assays, cells were again seeded (1–2 x $10^5$ cells/well) in 24-well tissue culture multiplates and grown overnight. The next day, cells were incubated with mixtures of a constant concentration of GpL-FLAG-TNC-TWEAK and increasing concentrations of FLAG-TWEAK for 1 h at 37 °C. Cell-associated luminescence activity of GpL-FLAG-TNC-TWEAK was then determined as described above for equilibrium binding studies.

Analysis of TWEAK dissociation from Fn14 was performed with cells that were grown overnight in 24-well tissue culture dishes (1–2 x $10^5$ cells/well). Initially, cells in half of the wells were again blocked with Fc-FLAG-TWEAK (1.5 μg/ml) for 30 min at 37 °C to determine nonspecific binding. Subsequently, cells were incubated with a constant concentration of GpL-FLAG-TNC-TWEAK for 1 h at 37 °C to reach equilibrium binding. Cells were then chased for varying times with an excess (1.5 μg/ml) of Fc-FLAG-TWEAK, and specifically bound GpL-FLAG-TNC-TWEAK activity was again determined as before.

To measure the kinetics of TWEAK-Fn14 association, cells (24-well plate; 1–2 x $10^5$ cells/well) were stimulated for increasing times with a constant concentration of GpL-FLAG-TNC-TWEAK, and bound luciferase activity was ascertained as described above. For determination of nonspecific binding, cells pretreated with Fc-FLAG-TWEAK (1.5 μg/ml; 30 min) were analyzed in parallel. Regression analysis of the data obtained and calculation of the kinetic parameters were performed with various functions of the GraphPad Prism 5 analysis software and are specified in the figure legends.

IL8 ELISA—Cells (HT1080 or HT29, 1 x $10^4$ cells/well) were grown overnight in 96-well tissue culture dishes. The next day, cell culture medium was changed to minimize the formation of inactive misfolded ligand species (26, 27). After 4–6 days of cultivation, cell culture supernatants of Hek293 cells stably transfected with expression plasmids containing various TWEAK and TNF variants for 6 h at 37 °C. The IL8 content of the supernatants was finally determined using the OptEIATM human IL8 set (Pharmingen) according to the protocol delivered by the manufacturer.

Silver Staining and Western Blotting—Protein samples were boiled in Laemmli sample buffer and separated by SDS-PAGE using a 12.5% gel. Proteins were visualized either by silver staining or by Western blotting. For the latter, proteins were transferred to a nitrocellulose membrane by semi-dry blotting. The blot was blocked with Tris-buffered saline containing 0.05% Tween 20 and 5% (w/v) dry milk to inhibit nonspecific antibody binding. Detection of the proteins of interest was performed using primary antibodies of the desired specificity, appropriate horseradish peroxidase-conjugated secondary antibodies (Dako, Hamburg, Germany), and the ECL Western blotting detection reagents and analysis system (Thermo Fisher Scientific, Munich, Germany).

Isolation of Monocytes—Human blood cells were initially obtained by density centrifugation of blood buffy coats with LSM 1077 lymphocyte separation medium (PAA Laboratories, Pasching, Austria). Monocytes were then isolated with the help of anti-CD14-conjugated magnetic beads and a MidiMACS Separator (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocyte isolation was considered as successful when FACS analysis of cell surface expression of CD14 resulted in >95% positive cells. Monocytes were then immediately used for FACS analysis of CD163 and Fn14 expression and binding studies with GpL-FLAG-TNC-TWEAK.

RESULTS AND DISCUSSION

N-terminal Fusion of G. princeps Luciferase Does Not Affect the Fn14 Stimulating Activities of Soluble TWEAK—The implementation of binding studies with chemically labeled TNF ligands (e.g. iodinated or biotinylated ligands) is typically aggravaed/limited by the poor reproducibility of the labeling process and particularly by the inherent risk to obtain mixtures of molecules that differ in activity and degree of labeling. Using CD95L as a prototypic representative of the TNF ligand family, we have recently evaluated the usefulness of various proteins (GpL (29), Metridia longa luciferase (MIL), yellow fluorescent protein (YFP), secreted alkaline phosphatase, and O6-alkylguanine-DNA-alkyltransferase-based SNAP tag) as reporter domains to overcome these limitations and to construct TNF ligand fusion proteins suitable for cellular binding studies. In this evaluation, GpL was not only found to be the most sensitive reporter domain but also did not interfere with the well established oligomerization dependence of activity of soluble CD95L trimers. Indeed, several of the other reporter domains tested, particularly YFP, secreted alkaline phosphatase, and MIL, resulted in CD95L variants with high oligomerization-independent activity, which is indicative of the presence of aggregated CD95L trimers. Thus, the GpL domain on the one hand did not affect the activity of the TNF domain in the CD95L fusion protein, and on the other hand it was highly traceable. GpL therefore appeared to us as especially useful for labeling of TNF ligands by genetic engineering. To generate a TWEAK probe useful for highly sensitive cellular binding studies, we therefore linked the N terminus of the THD of TWEAK to the C terminus of GpL. We included an internal FLAG epitope between the luciferase and the TWEAK domain to allow one-step purification by affinity chromatography (Fig. 1, A–C). We further included a small trimerization domain from tenascin C (~3 kDa) to stabilize the trimeric assembly of TWEAK and to minimize the formation of inactive misfolded ligand species (26, 27). After 4–6 days of cultivation, cell culture supernatants of Hek293 cells stably transfected with expression plasmids encoding secretable GpL-FLAG-TNC-TWEAK and FLAG-TWEAK contained typically 40–80 μg of the recombinant protein per 15-cm cell culture Petri dish. One-step purification of the recombinant TWEAK variants was possible with high recovery (>90%) by binding to anti-FLAG mAb M2-agarose and gentle elution with soluble FLAG peptide (data not shown). SDS-PAGE analysis and Western blotting revealed a major fuzzy anti-FLAG reactive molecular species for FLAG-TWEAK and a single band for GpL-FLAG-TNC-TWEAK migrating with molecular weights that are in good accordance with the theoretical molecular weight of the nonmodified proteins when

3 1. Lang and H. Wajant, manuscript in preparation.
moderate glycosylation is assumed (Fig. 1, B and C). In fact, glycosidase treatment resulted in a significantly faster migration of FLAG-TWEAK and a less fuzzy appearance of the corresponding band (Fig. 1D). In accordance with the expected trimeric assembly of GpL-FLAG-TNC-TWEAK, it eluted in gel filtration experiments in a single peak (Fig. 1E). With a standard luminometer, the lower detection limit of GpL-FLAG-TNC-TWEAK was below 0.1 pg/ml (1fM of the trimeric molecule), and its activity increased linearly with its concentration over more than 6 orders of magnitude (Fig. 1F).

To figure out whether the GpL domain of GpL-FLAG-TNC-TWEAK interferes with the biological activity of the TWEAK domain, we analyzed the functional properties of GpL-FLAG-TNC-TWEAK and FLAG-TWEAK in four different assays as follows: first, apoptosis induction in Kym-1 cells; second, stimulation of p100 processing in HT29 cells; third, inhibition of BMP2-induced alkaline phosphatase production; and fourth, up-regulation of IL8 synthesis in HT1080 cells. The first three cellular responses are efficiently triggered by soluble TWEAK irrespective of ligand oligomerization (20, 28). In contrast, with respect to IL8 production, oligomerized soluble TWEAK trimers and hexameric soluble TWEAK were superior to TWEAK trimers (Fig. 2D) (28).

In no case did we observe a major difference in the dose-response relationship of the four effects between GpL-FLAG-TNC-TWEAK and FLAG-TWEAK. Moreover, robust IL8 induction by GpL-FLAG-TNC-TWEAK required oligomerization in a similar fashion as FLAG-TWEAK (Fig. 2D), and responses triggered by GpL-FLAG-TNC-TWEAK were inhibited by ITEM-4, a blocking monoclonal antibody specific for Fn14 (Fig. 2E).

Equilibrium Binding of Human Soluble TWEAK Trimers to Cell-expressed Human and Murine Fn14—To initially confirm the cross-species specificity of human TWEAK for human and murine Fn14, Hek293 cells, which express comparatively low amounts of endogenous Fn14, were transiently transfected with expression constructs encoding human and murine Fn14 (Fig. 3A). Binding of GpL-FLAG-TNC-TWEAK to murine and human Fn14-transfected cells was more than 100-fold higher than binding to mock-transfected cells only expressing endogenous Fn14 (Fig. 3A). On the one hand, this confirmed the inter-species activity of human TWEAK, and on the other hand, this indicated that there is no major nonspecific binding of the construct. To investigate the TWEAK binding capability of human Fn14 in more detail, equilibrium binding studies with GpL-FLAG-TNC-TWEAK and HT29 and HT1080 cells were
performed at 37 °C. Fn14 expression was evident from FACS analysis for both cell lines (Fig. 3B). Nonspecific binding was determined by analyzing binding of GpL-FLAG-TNC-TWEAK to cells preincubated with an excess of Fc-FLAG-TWEAK. For binding studies, we regularly used concentrations between 3.2 and 1660 pM and 100–300,000 cells per sample. The obtained binding data fitted with high significance to one-site binding curves and revealed $K_m$ values of 94 pM for HT29 cells and of 76 pM for HT1080 cells (Fig. 3C and Table 1). Calculation of maximal binding sites resulted in 21,400 binding sites for HT29 cells and in 17,400 binding sites for HT1080 cells. At the lowest concentration (3.2 pM) used in our experimental standard setting, around 200–270 trimeric GpL-FLAG-TNC-TWEAK molecules were bound per cell. However, specific binding was typically >100-fold over background at this concentration. Thus, it was even possible to obtain specific binding (∼5-fold over background) at concentrations below 1 pM allowing the detection of less than 20 bound molecules per cell (data not shown). In a similar fashion, we obtained for murine Renca and B16 cells $K_D$ values of 112 and 53 pM for GpL-FLAG-TNC-TWEAK (Fig. 3C and Table 1). Noteworthy, Fn14 cell surface expression on B16 cells was evident from the cellular binding studies with GpL-FLAG-TNC-TWEAK and Fc-FLAG-TWEAK as competitor (Fig. 3C) but was not reliably detectable by standard FACS analysis (Fig. 3B). However, Fn14-specific antibodies blocked GpL-FLAG-TNC-TWEAK binding to B16 cells to a similar extent as the Fc-FLAG-TWEAK.4 Thus, the discrepancy in Fn14 detection using FACS and binding studies with GpL-FLAG-TNC-TWEAK is not due to an unknown binding partner of TWEAK on B16 cells but reflects the superior Fn14 traceability in the binding studies. The $K_D$ values

4 A. Fick, J. Trebing, and H. Wajant, unpublished results.
obtained from our cellular equilibrium studies are roughly in the same range as the concentration of 1 ng/ml (~18 pM) recombinant TWEAK required for half-maximal detection of immobilized recombinant Fn14 in Ref. 2. In the following, the $K_D$ value of the TWEAK-Fn14 interaction was also calculated from homologous competition assays with GpL-FLAG-TNC-TWEAK and FLAG-TWEAK on HT1080 and HT29 cells. In accordance with the values gained from the saturation binding experiments, this resulted in a $K_D$ value of 350 pM for HT1080 cells and of 170 pM for HT29 cells (Fig. 3D and Table 1). To determine the dissociation and association rate constants of the TWEAK-Fn14 interaction, dissociation was determined experimentally, and the dissociation rate constant obtained was then used together with the corresponding $K_D$ value to calculate the associated rate constant. To quantify TWEAK-Fn14 dissociation, HT1080 and HT29 cells were loaded with GpL-FLAG-TNC-TWEAK and were then chased with an excess of Fc-FLAG-TWEAK. The dissociation rate constant determined...
TWEAK-Fn14 Binding Studies

TABLE 1

| Cell line | \( K_D \) of GPL-FLAG-TNC-TWEAK\(^a\) \( \mu M \) | Binding sites per cell | \( K_D \) of FLAG-TWEAK\(^b\) \( \mu M \) | \( k_{\text{off}} \) \( s^{-1} \) | \( k_{\text{on}} \) \( s^{-1} \) | \( K_D = k_{\text{off}}/k_{\text{on}} \) \( \mu M \) |
|-----------|----------------|----------------------|----------------|----------------|----------------|----------------|
| HT1080    | 76 ± 6         | 17,400 ± 2000        | 350 ± 11       | 0.58 × 10\(^{-3}\) | 3.3 × 10\(^{6}\) | 180             |
| HT29      | 94 ± 41        | 21,400 ± 3300        | 170 ± 9        | 0.48 × 10\(^{-3}\) | 3.3 × 10\(^{6}\) | 150             |
| Renca     | 112 ± 9        | 25,200 ± 4300        | ND\(^c\)        | ND             | ND             | ND              |
| B16       | 53 ± 18        | 1500 ± 150           | ND\(^c\)        | ND             | ND             | ND              |

\(^a\) Data were derived from equilibrium binding study; \( n = 4 \).
\(^b\) Data were derived from homologous competition assays; \( n = 4 \).
\(^c\) \( n = 3–4 \) experiments.
\(^d\) \( n = 4 \) experiments.
\(^e\) ND means not determined.

induced much higher IL8 production than GPL-FLAG-TNC-TWEAK alone (Fig. 4, A and B). Remarkably, nonoligomerized GPL-FLAG-TNC-TWEAK induced weak but significant IL8 production, and the EC\(_{50}\) value of this response was close to the corresponding value of oligomerized GPL-FLAG-TNC-TWEAK (Fig. 4, A and B). Moreover, there was practically no difference in cellular binding of nonoligomerized and oligomerized GPL-FLAG-TNC-TWEAK after 6 h (Fig. 4, A and B), although the \( K_D \) values obtained after 6 h were regularly somewhat lower than those obtained in the standard 2-h binding assays. Future studies must show whether the increase in affinity with prolonged incubation is due to secondary processes, but in any case, the increased activity of TWEAK oligomers is definitely not related to higher Fn14 occupancy. Therefore, it has to reflect differences between individual TWEAK-Fn14 complexes and secondary oligomerized TWEAK-Fn14 complexes.

TNFR1 Has a Much Higher Intrinsic Capability than Fn14 to Stimulate Inflammatory Signaling—TWEAK, like the closely related cytokine TNF, triggers in a variety of cell lines proinflammatory pathways leading to the activation of the classical NF\(\kappa\)B pathway and various MAPKs. Moreover, the functional similarities of TWEAK and TNF \textit{in vitro} find their continuation \textit{in vivo}.

Both molecules have been implicated in regulation of innate immunity and play similar roles in a variety of animal models of autoimmune disease, including models for experimental autoimmune encephalomyelitis, collagen-induced arthritis, and 2,4,6-trinitrobenzene sulfonic acid-induced colitis (25, 32–36). Noteworthy, TNFR1, the major mediator of the proinflamma-

FIGURE 3. Binding studies with GPL-FLAG-TNC-TWEAK. A, Hek293 cells were electroporated with expression constructs encoding human and murine Fn14 or empty vector. After 2 days, cell surface expression of Fn14 was analyzed with the human/murine Fn14 cross-reactive mAb ITEM-4 by FACS (left panel). In parallel, cells (500 × 10\(^3\)) were washed and incubated in triplicate in suspension for 2 h with 25 ng/ml GPL-FLAG-TNC-TWEAK. After four washes in PBS, cell-associated luciferase activity was determined (right panel). B, indicated cell lines were analyzed by FACS with PE-labeled anti-Fn14 (ITEM-4) and a corresponding PE-labeled isotype control antibody. C, HT1080, HT29, B16, and Renca cells were seeded (2 × 10\(^5\) cells/well) in 24-well plates. After cultivation overnight, half of the cells were preincubated (1 h, 37 °C) with 1.5 μg/ml Fc-FLAG-TWEAK. Subsequently, cells were incubated (2 h, 37 °C) with the indicated concentrations of GPL-FLAG-TNC-TWEAK. After 10 washes with ice-cold PBS to remove unbound ligand, cells were collected in 50 μl of medium by scraping with a rubber policeman to determine cell-associated luciferase activity. Nonspecific binding was obtained from the samples pretreated with Fc-FLAG-TWEAK, and total binding was gained from the cells only treated with GPL-FLAG-TNC-TWEAK. Specific binding was calculated by subtracting nonspecific binding from the corresponding total binding values. Cells from one well were counted to allow calculation of receptor numbers per cell. Binding data were fitted with nonlinear regression to a one side specific binding plot using GraphPad Prism5 software to obtain \( B_{\max } \) and \( K_D \) values. One of four experiments summarized in Table 1 is shown for each cell line. D, homologous competition binding experiments with GPL-FLAG-TNC-TWEAK and FLAG-TWEAK. HT1080 and HT29 cells were incubated at 37 °C with mixtures of 25 ng/ml (200 pM) GPL-FLAG-TNC-TWEAK and the indicated concentrations of FLAG-TWEAK. After 1 h, luciferase activity of cell-bound GPL-FLAG-TNC-TWEAK was determined. One representative experiment is shown for each cell line. \( IC_{50} \) values were calculated using the GraphPad Prism5 software. E, HT1080 and HT29 cells were seeded (1 × 10\(^5\) cells/well) in a 24-well plate. Each plate, the cells in half of the wells were blocked for 0.5 h with an excess of conventional Fc-FLAG-TWEAK (2 μg/ml) to allow determination of nonspecific binding. Cells were incubated for 1 h with 25 ng/ml (200 pM) GPL-FLAG-TNC-TWEAK. To measure dissociation, 1.5 μg/ml Fc-FLAG-TWEAK was added for the indicated time intervals before cell-associated luciferase activity was determined. Specific binding was calculated for all time points as the difference of binding in samples with and without Fc-FLAG-TWEAK pretreatment. F, untreated and Fc-FLAG-TWEAK blocked (1.5 μg/ml) cells were stimulated with 32 (filled circles), 160 (open circles), and 800 (filled squares) pM of GPL-FLAG-TNC-TWEAK for the indicated times to determine the kinetics of the association of GPL-FLAG-TNC-TWEAK and Fn14. Association kinetics were fitted using GraphPad Prism5 software to obtain the association rate constant. PE, phycoerythrin; RLU, relative light units.
tory effects of TNF, is a member of the death receptor subgroup of the TNF receptor family, whereas Fn14 belongs to the TRAF protein-interacting TNF receptor group (37). Thus, both receptors obviously utilize different receptor-associated mechanisms to trigger activation of the aforementioned proinflammatory pathways. Thus, we next analyzed quantitatively the intrinsic capacity of TNFR1 and Fn14 to activate proinflammatory signaling by help of GpL-FLAG-TNC-TWEAK and a corresponding GpL variant of TNF. Similar as before for soluble TWEAK, addition of an N-terminal GpL domain (including the TNC stabilization domain) showed no major impact on the activity of FLAG-TNF. The corresponding fusion protein GpL-FLAG-TNC-TNF showed practically the same activity as conventional FLAG-TNC-TNF with respect to induction of the proinflammatory cytokine IL8 as well as with respect to apoptosis induction (Fig. 5, A and B). In particular, the GpL domain in GpL-FLAG-TNC-TNF elicited the same specific activity as the corresponding domain in GpL-FLAG-TNC-TWEAK (data not shown). In accordance with data from the literature obtained with iodinated TNF (38), binding studies with GpL-FLAG-TNC-TNF revealed a $K_D$ value of 24 pM for HT1080 cells and 47 pM for HT29 cells (Table 2 and Fig. 5C). To determine IL8 induction via TNFR1 and Fn14 as a function of the number of ligand-occupied active receptor molecules, we stimulated cells with GpL-FLAG-TNC-TNF and anti-FLAG oligomerized GpL-FLAG-TNC-TWEAK and measured IL8 production and ligand binding (Fig. 5D). In HT1080 cells, half-maximal IL8 production was achieved with an average of 27 GpL-FLAG-TNC-TNF trimers bound per cell, whereas 2690 cell-bound GpL-FLAG-TNC-TWEAK trimers were required to elicit the same response. Thus, the average IL8-inducing activity of an activated TNFR1 complex appeared ~100-fold higher than those of an activated Fn14 complex. Similar results were also obtained when HT29 cells were analyzed for IL8 induction (Fig. 5E). Half-maximal IL8 induction by TNF required 24 cell-bound ligand trimers, although 4250 cell-bound aggregated TWEAK trimers were required. The much lower intrinsic activity of anti-FLAG-oligomerized GpL-FLAG-TNC-TWEAK-stimulated Fn14 and TNFR1 activated by GpL-FLAG-TNC-TNF is unlikely related to insufficient oligomerization of Fn14-bound GpL-FLAG-TNC-TWEAK because anti-FLAG-oligomerized FLAG-TWEAK is comparably active as the Fc-TWEAK variant forming highly active hexameric molecules (28).

Cell surface expression of TNFR1 is typically in the range of 300–2000 receptors per cell and is thus 1 to 2 orders of magnitude lower than those of Fn14. It is therefore tempting to speculate that the comparatively high expression of Fn14 serves to circumvent its low intrinsic ability to activate proinflammatory pathways such as the classical NFkB pathway. Notably, the ability of Fn14 to stimulate the classical NFkB pathway is not directly related to TRAF2 recruitment and activation of the alternative NFkB pathway. So, Fn14 strongly interacts with TRAF2 irrespective of oligomerization of soluble TWEAK trimers, and in contrast to the classical NFkB pathway p100 processing is induced by TWEAK trimers and TWEAK oligomers with the same efficacy (28). Thus, it appears that activation of the classical NFkB pathway requires some kind of trans-interaction of TRAF2 complexes recruited to trimeric TWEAK-Fn14 complexes, whereas the sole recruitment to Fn14 is fully sufficient for p100 processing. In line of this, we...
and others have shown that the deviation of cytosolic TRAF2-cIAP1/2 complexes is already sufficient to withdraw NFκB-inducing kinase from the inhibitory action of this complex leading to NFκB-inducing kinase accumulation and activation of the alternative NFκB pathway (28, 30). The efficacy of deviation of TRAF2-cIAP1/2 complexes is obviously foremost a function of the cell type.

FIGURE 5. TNFR1 has a much higher capability than Fn14 to stimulate the classical NFκB pathway. A, HT1080 cells (10 × 10³ cells/well, 96-well plate) were stimulated in triplicate with increasing concentrations of FLAG-TNC-TNF and GpL-FLAG-TNC-TNF for 6 h, and supernatants were analyzed by ELISA for their IL8 content. Background caused by constitutive IL8 production was minimized by changing the cell culture medium prior to stimulation. B, Kym-1 cells (10 × 10³ cells/well, 96-well plate) were stimulated in triplicate with the indicated concentrations of the two TNF variants, and after 16 h remaining cellular viability was determined by staining with crystal violet. C, HT1080 and HT29 cells were seeded (10 × 10⁴ cells/well) in 24-well plates. The next day, half of the cells were blocked (1 h, 37 °C) with 10 μg/ml of the TNFR1-specific antibody H398. Cells were then incubated (2 h, 37 °C) with increasing amounts of GpL-FLAG-TNC-TNF, and after 10 washes with ice-cold PBS cells were collected in 50 μl of medium by scraping with a rubber policeman. Nonspecific binding was obtained from the H398-blocked samples and subtracted from total binding of the cell samples only treated with GpL-FLAG-TNC-TNF to calculate specific binding. D and E, HT1080 (D) and HT29 (E) cells were seeded (1 × 10⁵ cells/well) in 24-well plates. The next day, cells were blocked for 1 h at 37 °C with 1.5 μg/ml of Fc-FLAG-TWEAK, 10 μg/ml of the TNFR1 blocking mAb H398, or remained untreated. Cells were then stimulated with the indicated concentrations of the GpL domain-tagged variants of FLAG-TNC-TWEAK and FLAG-TNC-TNF. To allow optimal IL8 induction via Fn14, GpL-FLAG-TNC-TWEAK was oligomerized with anti-FLAG (1 μg/ml M2). Supernatants of the nonblocked samples of each of the two ligands were collected after 6 h to determine the IL8 production by ELISA. The amount of cell-associated ligands was determined by measuring luciferase activity. The difference between total binding of GpL-FLAG-TNC-TWEAK or GpL-FLAG-TNC-TNF and the corresponding nonspecific binding (Fc-FLAG-TWEAK and H398-pretreated samples) was considered as specific binding. RLU, relative light units.
of Fn14 affinity and directly correlates with the number of liganded Fn14 molecules, although the intrinsic trans-activity of the Fn14 signaling complex necessary to trigger the classical NFkB pathway here is presumably secondary. This idea could easily explain why soluble TWEAK is a strong inducer of p100 processing despite having only a low intrinsic capacity to stimulate classical NFkB signaling. In line with this, the low expression levels of TNFR1 and thus its low capability to deviate cytosolic TRAF2-cIAP1/2 complexes deliver a rationale of why this receptor fails to trigger p100 processing despite being highly efficient in the recruitment of TRAF2. In case of stimulation of Fn14 with membrane TWEAK or oligomerized TWEAK, there is further evidence that Fn14-mediated depletion of TRAF2-cIAP1/2 complexes is enhanced/sustained by proteolytic degradation of TRAF2 (30, 31), whereas in the context of TNFR1 signaling TRAF2 degradation is blocked by RIPK1 (39, 40). Moreover, there is some initial evidence that TWEAK is able to trigger p100 processing by a yet poorly understood cIAP1- and cIAP2-independent pathway (41).

### Table 2

| Cell line | $K_D$ of GpL-FLAG-TNC-TNF | Binding sites per cell |
|-----------|--------------------------|-----------------------|
| HT1080    | 24 ± 5                   | 405 ± 49              |
| HT29      | 47 ± 14                  | 739 ± 69              |

*Data were derived from equilibrium binding study; $n = 4$.

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**FIGURE 6. Analysis of interaction of TWEAK and CD163.** A, Hek293 cells were electroporated with an expression construct encoding human CD163 or empty vector. The next day, CD163 cell surface expression was analyzed by FACS (upper panel), and in parallel cells (500 × 10^3) were incubated in triplicate in suspension for 2 h with 25 ng/ml GpL-FLAG-TNC-TWEAK. Prior to GpL-FLAG-TNC-TWEAK incubation, cells were treated with FLAG-TWEAK (6 µg/ml) or ITEM-4 (2 µg/ml). After four washes in PBS, cell-associated luciferase activity was determined (lower panel). B, monocytes were purified from peripheral blood mononuclear cells by magnetic activated cell sorting separation and were immediately analyzed by FACS with respect to CD163 and Fn14 expression (upper panel). In parallel, binding studies as described in A were performed (lower panel). C, Fn14-Fc was immobilized on 96-well plastic plates. After blocking with FCS, wells were preincubated in triplicate with 1 µg/ml recombinant CD163 (rCD163), ITEM-4 (1 µg/ml), and FLAG-TWEAK (1 µg/ml) and binding of GpL-FLAG-TNC-TWEAK (10 ng/ml) was analyzed. D, Hek293 cells were transiently transfected by electroporation with full-length TWEAK or FLAG-TWEAK, and supernatants were collected 5 days post-transfection and analyzed by Western blotting with respect to processed soluble TWEAK using TWEAK-specific (left panel, upper part) and FLAG-specific (left panel, lower part) antibodies. FLAG-TWEAK and cell-derived soluble TWEAK were treated with recombinant CD163 (5 µg/ml) and then used to challenge Kym-1 cells. The next day, cell viability was determined using crystal violet staining (right panel). A group with cells pretreated with ITEM-4 (5 µg/ml) was included to confirm the Fn14 dependence of TWEAK-induced apoptosis.
analyzed the transfectants with respect to GpL-FLAG-TNC-TWEAK binding. To differentiate between binding of GpL-FLAG-TNC-TWEAK to CD163 and GpL-FLAG-TNC-TWEAK to endogenous Fn14, we determined nonspecific binding by pretreatment of the cells with an excess of FLAG-TWEAK, which should block both interactions as well as by preincubation with the anti-FLAG mAb ITEM-4, which only blocks the TWEAK-Fn14 interaction. Surprisingly, we got no evidence for an interaction between CD163 and GpL-FLAG-TNC-TWEAK from these experiments (Fig. 6A). First, total binding of GpL-FLAG-TNC-TWEAK to CD163 and empty vector transfected cells was indistinguishable, and second, both ITEM-4 and FLAG-TWEAK blocked total binding of GpL-FLAG-TNC-TWEAK to the same extent for more than 95% (Fig. 6B). Moreover, we failed to observe an inhibitory effect of recombinant soluble CD163 on binding of GpL-FLAG-TNC-TWEAK to immobilized Fn14-Fc (Fig. 6C). To rule out the lack of evidence for a CD163-TWEAK interaction in our binding studies is related to an interference of the GpL-FLAG-TNC part of our TWEAK fusion protein with CD163 binding, we performed a functional assay where we looked for an inhibitory effect of recombinant soluble CD163 on soluble TWEAK released from cells expressing full-length membrane TWEAK. For this purpose, we analyzed Fn14-mediated apoptosis in Kym-1 cells, which is readily inducible by nonoligomerized soluble TWEAK trimers (Fig. 2A) (20). Cell culture supernatants containing FLAG-TWEAK as well as membrane TWEAK-derived soluble TWEAK triggered cell death in Kym-1 cells, and in both cases this was largely inhibitable by the Fn14 blocking mAb ITEM-4 (Fig. 6D). However, pretreatment of the TWEAK containing supernatants with recombinant CD163 showed no effect on cell death induction (Fig. 6D). In sum, our experiments supply no evidence for an interaction of CD163 and TWEAK at biologically meaningful concentrations.

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