A novel llama antibody targeting Fn14 exhibits anti-metastatic activity in vivo

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Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; cIAP1/2, cellular inhibitor of apoptosis-1/2; eGFP, enhanced green fluorescent protein; Fn14, fibroblast growth factor (FGF)-inducible 14; FcgR, Fcγ receptor; IL8, interleukin-8; NfkB, nuclear factor kappa-B; NIK, NfkB inducing kinase; TNFR1, tumor necrosis factor (TNF) receptor-1 (TNFR1); TRAF, TNF receptor associated factor; TWEAK, tumor necrosis factor (TNF)-like weak inducer of apoptosis

Expression of fibroblast growth factor (FGF)-inducible 14 (Fn14), a member of the tumor necrosis factor receptor superfamily, is typically low in healthy adult organisms, but strong Fn14 expression is induced in tissue injury and tissue remodeling. High Fn14 expression is also observed in solid tumors, which is why this receptor is under consideration as a therapeutic target in oncology. Here, we describe various novel mouse-human cross-reactive llama-derived recombinant Fn14-specific antibodies (5B6, 18D1, 4G5) harboring the human IgG1 Fc domain. In contrast to recombinant variants of the established Fn14-specific antibodies PDL192 and P4A8, all three llama-derived antibodies efficiently bound to the W42A and R56P mutants of human Fn14. 18D1 and 4G5, but not 5B6, efficiently blocked TNF-like weak inducer of apoptosis (TWEAK) binding at low concentrations (0.2–2 µg/ml). Oligomerization and Fcγ receptor (FcγR) binding converted all antibodies into strong Fn14 agonists. Variants of 18D1 with enhanced and reduced antibody-dependent cell-mediated cytotoxicity (ADCC) activity were further analyzed in vivo with respect to their effect on metastasis. In a xenogeneic model using human colon carcinoma cancer cells, both antibody variants were effective in reducing metastasis to the liver. In contrast, only the 18D1 variant with enhanced ADCC activity, but not its ADCC-defective counterpart, suppressed lung metastasis in the RENCA model. In sum, this suggests that Fn14 targeting might primarily act by triggering of antibody effector functions, but also by blockade of TWEAK-Fn14 interaction in some cases.

Introduction

Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) and its receptor Fn14 belong to the TNF ligand and the TNF receptor family, respectively. Like other ligands of the TNF family, TWEAK is a membrane-bound type II protein, but membrane TWEAK is only rarely detectable, e.g., on monocytes and macrophages, due to efficient processing to a soluble molecule by furin proteases.¹,² Despite the capability of TWEAK to trigger cell death in some cellular models (hence its name), Fn14 does not recruit death domain-containing proteins, and instead interacts with E3 ligase/adaptor proteins of the TNF receptor associated factor (TRAF) family.¹,³ In fact, the apoptosis-inducing activity of the TWEAK/Fn14 system has been traced back to an indirect mechanism: the induction of TNF and subsequent stimulation of the death receptor TNF receptor-1 (TNFR1) along with sensitization for TNFR1-induced apoptosis by inhibition of anti-apoptotic complexes composed of TRAF2 and cellular inhibitor of apoptosis-1 (cIAP1) and/or cIAP2.³,⁵ It is worth mentioning that Fn14 adopts different states of signaling activity depending on whether membrane-bound or soluble TWEAK activate it. While both TWEAK forms are equally effective in stimulating the alternative nuclear factor kappa-B (NFκB) pathway, and in cell death induction, and in enhancement of TNFR1-mediated cell death, membrane TWEAK is superior in the activation of the classical NfkB pathway.⁶ Moreover, in the absence of FcγR binding, Fn14-specific antibodies affect Fn14-related signaling activities in a manner distinct from both TWEAK forms. They trigger strong p100 processing in some cell lines, such as HT29 and WiDr cells, but neither enhance TNFR1-induced cell death nor activate the classical NfkB pathway and may even block the stimulation of these cellular responses to soluble and membrane TWEAK.⁷
Fn14 is strongly and dynamically expressed during early stages of development, but typically only low expression of this receptor occurs in the adult healthy organism.\textsuperscript{1,2} In accordance with studies showing Fn14 expression to be controlled by growth factors and various cytokines, there is, however, rapid and robust induction of Fn14 in response to tissue damage.\textsuperscript{1,2} In line with this, the TWEAK/Fn14 system elicits pleiotropic functions in tissue remodeling and tissue repair, including the stimulation of proliferation and differentiation of mesenchymal progenitor cells, triggering of angiogenesis, and recruitment of immune cells.\textsuperscript{1,2} Although the latter cell types appear to be the main source of membrane TWEAK, the soluble form of the ligand might also be produced by activated resident cells. As tumors and their stroma are recognized as "wounds that do not heal,"\textsuperscript{8} it is not surprising that most solid tumors exert strong Fn14 expression. The latter is evident both on non-malignant cells of the tumor microenvironment and on the transformed cells. Indeed, Fn14 is strongly expressed in vitro by most tumor cell lines of non-lymphoid origin.

Although the TWEAK/Fn14 system might primarily regulate tissue homeostasis and wound healing in the healthy organism, it appears to convert to a disease driving/maintaining factor in scenarios characterized by exaggerated and chronic tissue damage. Analysis of Fn14- and TWEAK-deficient mice along with studies exploiting TWEAK-neutralizing proteins demonstrated a crucial role of the TWEAK/Fn14 system in various models of autoimmune/inflammatory diseases, including myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis, collagen induced arthritis, 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis, in autoimmune encephalomyelitis, collagen induced arthritis, oligodendrocyte glycoprotein (MOG)-induced experimental autoimmun encephalomyelitis, collagen induced arthritis, 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis, in acute kidney injury, chronic and systemic lupus erythematosus (SLE)-related nephritis, and muscle injury.\textsuperscript{1,2,9-13} Moreover, TWEAK and Fn14 drive harmful inflammatory and fibrotic processes in response to liver or muscle damage, stroke, and renal and cerebral ischemia.\textsuperscript{1,2,13,14} The pathophysiological relevance of TWEAK and Fn14 and the tumor-associated expression of Fn14 provide the rationale to target these molecules in the aforementioned diseases and in cancer therapy. In fact, BIIB-023, a TWEAK-specific antibody, has been evaluated in a Phase 1 study in patients suffering from rheumatoid arthritis and showed a good tolerance and safety profile (ref. 15; http://www.clinicaltrials.gov; identifier: NCT00771329). BIIB-023 is now under consideration in a Phase 2 study in patients with lupus nephritis (http://www.clinicaltrials.gov; identifier: NCT01499355). Furthermore, RO-5458640, another TWEAK-specific antibody, and PDL192, a Fn14-specific antibody, have been investigated in Phase 1 studies in patients with advanced solid cancers (http://www.clinicaltrials.gov; identifiers: NCT01383733 and NCT00738764, respectively).

It is noteworthy that initial evidence supports not only inhibition of TWEAK/Fn14 as a potential therapeutic option, but also exogenous Fn14 stimulation, e.g., to protect neurons, to antagonize TNF-induced insulin resistance or to improve regeneration of β-cells or liver regeneration.\textsuperscript{16-19}

Based on the isolation of Fn14-specific camelid-derived antibodies, we describe here the development of chimeric mouse-human cross-reactive Fn14-specific IgG1 monoclonal antibodies (mAbs) that efficiently block TWEAK-induced Fn14 signaling. Like other Fn14-targeting antibodies, our novel llama antibodies convert into highly active Fn14 agonists upon oligomerization or Fcy receptor (FcyR) binding. Comparing antibody-dependent cell-mediated cytotoxicity (ADCC)-enhanced and ADCC-defective variants of one of these anti-Fn14 antibodies, we observed ADCC-dependent anti-metastatic activities in two cancer models, whereas the ADCC-defective variant was found to be effective in only one model. Thus, Fn14 antibody targeting might elicit anti-metastatic effects not only by stimulation of antibody dependent effector functions, but also by interfering with TWEAK-induced Fn14 activation.

Results

Generation of cross species-reactive Fn14-specific antibodies

Llamas were repeatedly immunized with Fn14-expressing cells or recombinant Fn14-Fc, and antibody phage-display libraries were constructed from peripheral blood cells. After three rounds of selection on immobilized Fn14-Fc, Fabs were prepared from the enriched phage populations and analyzed for the capability to block binding of Flag-TWEAK to Fn14-Fc. Various antibodies representing 20 distinct VH families were identified and expressed as human IgG1. Based on high affinity and cross-specificity for human and murine Fn14, three antibodies, 4G5, 18D1, and 5B6, were selected for more detailed characterization. We also included in our studies human IgG1 versions of the Fn14-specific, mouse-derived antibodies P4A8\textsuperscript{20,21} and PDL192.\textsuperscript{22} Taking into account the high structural conservation of Fn14, we evaluated whether the Fn14 mAbs might bind to different epitopes. To do this, we transiently expressed in HEK293 cells murine, cynomolgus, and human Fn14 along with two mutants of the latter (W42A and R56P) that are differentially recognized by P4A8 and PDL192 and assayed the transfectants for antibody binding by FACS. HEK293 cells have comparably low endogenous Fn14 expression and orders of magnitude higher receptor expression was reached by electroporation with Fn14 variant-encoding expression plasmids (Fig. 1A). All antibodies tested allowed effective staining of human and cynomolgus Fn14 (Fig. 1B). There were, however, quite distinct staining patterns with respect to murine Fn14 and the two human Fn14 mutants. With the exception of PDL192, all antibodies reacted well with murine Fn14 and the R56P mutant. The W42A variant was stained by PDL192 and the three llama-derived mAbs, but not by P4A8 (Fig. 1B). In sum, this suggests that PDL192, P4A8, and the three llama-derived mAbs bind to at least three different epitopes of Fn14.

The anti-Fn14 mAbs 4G5 and 18D1 efficiently block TWEAK-induced cellular reactions

Next, we investigated the anti-Fn14 antibody panel with respect to agonism and antagonism on Fn14-related cellular effects. A major effect of TWEAK is the induction of proinflammatory proteins via the NFκB system. We thus compared the effect of the various antibodies on TWEAK-induced cytokine production. As shown earlier,\textsuperscript{9} Flag-TWEAK trimers
oligomerized with the anti-Flag mAb M2, which mimic the activity of membrane TWEAK, efficiently triggered this response in HT1080 cells (Fig. 2A). In contrast, all antibodies failed to significantly stimulate IL8 production up to concentrations of 5 µg/ml (Fig. 2A). Preincubation of the HT1080 cells with the Fn14-specific antibodies, however, resulted, in case of P4A8, 18D1, and 4G5, in strong and complete inhibition of Flag-TWEAK/M2-induced IL8 production (Fig. 2B). In contrast, PDL192 and 5B6 showed only a very minor inhibitory effect at the highest concentrations used. Similar results were obtained when the Fn14 antibodies were analyzed in a model of TWEAK-induced cell death. While Fc-TWEAK triggered cell death of SKOV-3 cells, none of the Fn14-specific antibodies did so by themselves (Fig. 2C). TWEAK-induced cell death, was again completely blocked by P4A8, 18D1, and 4G5, while PDL192 and 5B6 showed only a moderate inhibitory effect at high concentrations (Fig. 2D). In accordance with the poor inhibitory effect of 5B6 on TWEAK/M2-induced cellular
Figure 2. For figure legend, see page 301.
responses, we further found 5B6 to be less effective in blocking the binding of GpL-TNC-Flag-TWEAK, a luciferase fusion protein of TWEAK, to Fn14 than 4G5 and 18D1 (data not shown).

We recently demonstrated that the terms agonistic and antagonistic for Fn14 targeting reagents must be used with caution because different pathways respond differentially to one and the same Fn14-targeting reagent.7 Especially, we showed in HT29 cells that PDL192 and P4A8 fail to stimulate the majority of Fn14-associated pathways but nevertheless nicely trigger p100 processing and NFκB inducing kinase (NIK) accumulation, two hallmarks of the alternative NFκB pathway. Thus, although P4A8 act as an inhibitor of TWEAK-induced cell death and IL8 production, it is concomitantly able to trigger the alternative pathway and thus has an activity pattern distinct from soluble and membrane TWEAK. These findings were also confirmed here for all three camelid-derived antibodies (Fig. 2E).

Fn14-specific IgG1 antibodies become strong agonists upon oligomerization or Fcγ receptor binding

In a few experiments using very high antibody concentrations (5–50 µg/ml), we observed moderate but significant IL8 induction (data not shown). This pointed to a minor residual agonistic activity of the antibodies or to the presence of aggregated antibody species with agonistic activity. Indeed, oligomerization with protein G or binding to FcRs can achieve strong agonistic activity for various antibodies targeting members of the TNF receptor family. We have recently shown a similar behavior with P4A8 and PDL192 and, accordingly, all three Fn14-targeting llama antibodies displayed strong agonistic activity upon crosslinking with protein G and efficiently triggered interleukin-8 (IL8) production, cell death induction and enhancement of TNF-induced cell death (Fig. 3A-C). To further test whether 4G5, 18D1, and 5B6 can acquire agonistic activity by interaction with Fc receptors, we analyzed co-cultures of HT1080 cells and HEK293 cells transiently expressing CD16, CD32A CD32B, and CD64 and analyzed anti-Fn14 antibody-induced IL8 production. As observed before for PDL192 and P4A8, all three llama-derived antibodies acted as strong agonists in the presence of Fcγ receptor expressing cells (Fig. 3D).

Generation of 18D1 variants with enhanced and reduced ADCC activity

Next, we addressed the potential relevance of different effector mechanisms of anti-Fn14 antibodies for their activity in vivo. While blockade of TWEAK binding requires no intact Fc domain, Fn14 activation and triggering of antibody-dependent immune effector mechanisms, such as ADCC, are dependent on Fcγ receptor binding. The latter two effects thus need a functional Fc domain and the presence of immune effector cells, e.g., natural killer (NK) cells, monocytes or macrophages. Furthermore, it must be considered that anti-Fn14 effects related to the inhibition of TWEAK binding might be compensated or even overcompensated by FcγR binding-mediated gain of agonistic activity. Depending on the particular pathological situation, both inhibition and engagement of Fn14 could be a therapeutic option. This also holds true in the context of a tumor therapeutic background where all the aforementioned effects of anti-Fn14 antibodies could in sum result both in an antitumoral response and in pro-tumoral effects. Thus, to initially delineate different effects caused by anti-Fn14s in vivo, we generated Fc domain mutants of 18D1 with enhanced (S239D, L332E; ref. 23) and diminished (E233P, L234V, L235A; ref. 24) FcγR binding. The 18D1 variants with the mutated Fc domains showed unchanged Fn14 binding and thus had the same inhibitory activity on TWEAK-induced IL8 production as the wild type 18D1 molecule (Fig. 4A). More important, the 18D1-dead variant completely failed to trigger an IL8 response in co-cultures with CD16 transfectants and furthermore showed significantly reduced Fn14 stimulation in the presence of CD32A and CD32B. CD64-dependent activation of Fn14, however, remained unaffected (Fig. 4B). In accordance with the central role of CD16 in NK cell-mediated ADCC, in vitro analysis proved that 18D1-enhanced and 18D1-dead greatly differ in their ability to trigger ADCC (Fig. 4C).

Tumor model-dependent anti-metastatic effects of 18D1-enhanced and 18D1-dead

To evaluate the potential of 18D1 to elicit ADCC-dependent and ADCC-independent anti-metastatic effects in mice, we tested 18D1-enhanced along with 18D1-dead in three cancer models: (1) a syngeneic model of lung metastasis with RENCA cells, (2) an intraportal injection xenotransplant model of hepatic metastasis with HCT116 cells, and (3) a bone metastasis model with syngeneic myeloma MOPC-315 cells. All three cell lines used in these animal models showed no cell death induction in response to recombinant TWEAK in vitro (data not shown). This also applied to these cells when they were sensitized with interferon-γ (IFNγ), which is typically produced by immune cells of the tumor microenvironment, and can sensitize some cell lines for TWEAK-induced cell death.25,26 In accordance with the well-established growth arrest-inducing activity of IFNγ, all IFNγ-treated cells showed somewhat reduced viability values. However, there was no further significant reduction in viability when the cells were additionally stimulated via Fn14.
While RENCA and HCT116 cells are Fn14-positive, MOPC-315 cells lack Fn14 expression. Syngeneic mice that were challenged with $10^5$ RENCA cells and treated with a control IgG1 antibody developed lung metastases with an incidence of 50% (8/16) within three to four weeks. When mice were treated twice per week starting one day after tumor cell application, 18D1-dead showed no significant effect on metastasis (9/15) while 18D1-enhanced almost completely.
osteoblast differentiation. For the MOPC-315 model we bone destruction and induces sclerostin, a major inhibitor of this model in our initial studies as TWEAK drives inflammatory Although MOPC-315 cells do not express Fn14, we included this model in our initial studies as TWEAK drives inflammatory bone marrow- crucially contributes to the antitumoral effect of 18D1 in this model. The MOPC-315 model shows strong bone marrow-homing upon intravenous injection of the myeloma cells. The MOPC-315 cells do not express Fn14, we included this model in our initial studies as TWEAK drives inflammatory bone destruction and induces sclerostin, a major inhibitor of osteoblast differentiation. For the MOPC-315 model we used again a luciferase transduced variant (MOPC-315.BMP2, FUGLW) that allowed non-invasive tracking of the tumor cells by whole body bioluminescence imaging. In the MOPC-315. BMP2.FUGLW model, neither 18D1-dead nor 18D1-enhanced showed a therapeutic effect (Fig. 5D). This suggests that MOPC-315.BMP2.FUGLW cells trigger the creation of a permissive bone marrow niche largely independent from the activity of the TWEAK/Fn14 system. Future studies must now elucidate in more detail the cellular mechanisms underlying the anti-metastatic effect of the two 18D1 variants in the RENCA and HCT116 model and will furthermore reveal whether 18D1 can also elicit anti-metastatic effects when treatment occurs only at later stages of metastasis.

Discussion

Using a phage-display library derived from peripheral blood cells of llamas immunized with Fn14-expressing cells or Fn14-Fc, we isolated a panel of Fn14-specific antibodies from which three were used to construct human IgG1 variants. Against the background of the high degree of similarity in the variable domains of llama and human antibodies, the llama anti-Fn14 human IgG1 chimera obtained by genetic engineering practically represent humanized antibodies (Table 1). Three of the llama-derived antibodies (5B6, 18D1, 4G5) with high affinity to Fn14 and varying capabilities to block TWEAK binding were expressed as hlgG1 molecules and analyzed in comparison to PDL192 and P4A8, two anti-Fn14 human IgG1 antibodies currently under investigation in clinical trials. Comparative analyses of the binding of the llama-derived anti-Fn14 antibodies, PDL192 and P4A8 to Fn14 suggest that the latter two and the llama-derived antibodies interact with at least partly non-overlapping epitopes on Fn14 (Fig. 1A). While 18D1 and 4G5 were as effective in blocking TWEAK-induced activation of Fn14 as was P4A8, 5B6 rather resembled in this respect PDL192 and thus showed no major inhibitory effect (Fig. 2). Noteworthy, all antibodies investigated showed at best a moderate agonistic effect at high concentrations of 5–10 μg/ml, but they all acted as strong Fn14 agonists upon oligomerization or binding to FcγRs (Fig. 3). Thus, the development of blocking anti-Fn14 antibodies for clinical applications may only succeed when Fc receptor binding can be avoided, e.g., by genetic engineering of the Fc domain. We recently observed that PDL192 and P4A8 triggered p100 processing and NIK accumulation, two hallmarks of the alternative NFκB pathway, in some cell lines (e.g., HT29 and WiDr cells), but failed to show a stimulating effect on other TWEAK/Fn14-mediated effects such as induction of proinflammatory cytokines or apoptosis and enhancement of TNF-induced cell death. We also observed such an alternative NFκB pathway-specific agonistic activity with all three llama-derived antibodies, arguing that this reflects a general property of anti-Fn14s. The in vivo relevance of this effect is, however, yet unclear.

In vitro, most tumor cells are resistant against cell death-induction by TWEAK or anti-Fn14 antibodies. This suggests that the strong in vivo anti-tumor effects observed with Fn14-specific antibodies mainly rely on the activation of Fc effector mechanisms. Indeed, an aglycosylated variant of BIB036 and the murine IgG1 variant of 19.2.1, the murine parental antibody of the humanized PDL192 antibody, have a reduced FcγR binding capacity and showed significantly reduced anti-tumor effects compared with their human IgG1 counterparts. We made a similar observation in the RENCA lung metastasis model where an ADCC-enhanced version of 18D1 efficiently inhibited metastasis while a corresponding ADCC-dead variant showed no effect at all (Fig. 5B). It is noteworthy that, in the intraportal injection liver metastasis model with the colon cancer cell line HCT116, both variants of 18D1 elicited a metastasis inhibitory effect (Fig. 5C). This resembles the situation that has been described for 19.2.1 in the A357 model where the ADCC-competent murine IgG2a variant and the ADCC-“low competence” murine IgG variant showed a comparable growth inhibitory effect in vivo. As A357 cells undergo cell death in response to Fn14 stimulation, but PDL192/19.2.1 practically does not interfere with TWEAK-Fn14 interaction, 19.2.1 seems to act in the A357 model by triggering Fn14-mediated cell death. In accordance with this idea, we observed that the sole binding to any type of FcγR converted PDL192 into a highly potent Fn14 agonist (ref. 7 and Fig. 3). The 18D1-dead variant used in this study also has residual FcγR binding properties, and one can thus
not definitely rule out that the suppression of HCT116 metastasis by 18D1-dead is based on Fn14 activation. HCT116 cells, however, are highly resistant against TWEAK and TWEAK/TNF mixtures in vitro. Thus, it is tempting to speculate that 18D1-dead inhibit HCT116 metastasis by blocking ligand binding of Fn14. Although it remains to be clarified in future studies whether both 18D1 variants act by a common mechanism or whether different mechanisms are in operation, e.g., Fn14 blockade in case of 18D1-dead and ADCC in case of 18D1-enhanced, this suggests that Fn14 blockade can be efficient in the absence of FcγR binding. The evidence that sole blockade of Fn14 can have an anti-tumor/metastatic effect is especially important in view of first results of clinical trials with PDL192 that show relatively low dose limiting toxicity at doses of 1–1.5 mg/kg. 30 Although the underlying mechanisms for the observed side effects are not resolved yet, stimulation of Fn14-mediated inflammatory effects by FcγR-bound anti-Fn14 or directing cytotoxic immune effector functions to off-target bound anti-Fn14 are straight-forward explanations.

In summary, we propose that targeting Fn14 with Fc crippled blocking antibodies that avoid the induction of therapy limiting side effects may pose a therapeutic option for a subset of tumor types.

**Material and Methods**

**Cell lines and reagents**

RENCA, HCT116-luc, HT1080, SKOV3, MOPC-315, BMP2,FUGLW, and HT29 cells were grown in RPMI1640 medium (PAA, Pasching, Germany) supplemented with 10% fetal calf serum (FCS; PAA). Production, purification, and functional evaluation of Flag-TWEAK and Fc-TWEAK are described elsewhere.6 Recombinant PDL192 and P4A8 have been generated by genetic engineering as described elsewhere7 by help of publicly-available amino acid sequences of the corresponding variable domains31,32. Protein G was purchased from Calbiochem, pCMV-SPORT6 expression vectors for C16, CD32A, CD32B, and CD64 were from Source Bioscience, D-luciferin was from Biosynth, and

![Graphs](image-url)
To achieve transient expression. For this purpose, 40 × 10^6 cells resulting in "Lambda" and "Kappa" llama Fab-libraries with libraries were then re-cloned in the VHCH1-containing library independently constructed in pCB3 by PCR. The light chain plasmids encoding the protein of interest (Fn14 variants, FcγRs) served to enrich for blocking antibodies. Fn14-interacting proteins A and by gel filtration.

Generation of llama-derived anti-Fn14 antibodies

Llamas (Ardèche Lamas, Saint-Remèze, France) were immunized with Fn14-expressing cells (786-O, Fn14 transfected HEK293) or Fn14-Fc. Peripheral blood lymphocytes were isolated from the immunized animals and used to generate a Fab-encoding gene segment phagemid library as described by de Haard et al.33 In brief, VλCλRs in co-cultures with HT1080 cells were used to enrich for blocking antibodies. Fn14-interacting clones were individually constructed in pCB3 by PCR. The light chain libraries were then re-cloned in the VHCH1-containing library resulting in “Lambda” and “Kappa” llama Fab-libraries with approximately 5–14 × 10^5 clones. To select for Fn14-interacting Fabs, phages were allowed to bind for 2 h to 96-well microtiter plates coated with Fn14-Fc. After repeated washing steps, phages were eluted with trypsin or recombinant TWEAK. The latter was used to enrich for blocking antibodies. Fn14-interacting clones were individually expanded in a 96-well deep well plate and periplasmic fractions were assayed (ELISA, Biacore) for Fabs binding to human or mouse Fn14-Fc. Several clones were converted into complete IgG1 antibodies by genetic fusion with the constant part of human IgG1, produced in HEK293 cells by transient expression and purified by affinity chromatography on protein A and by gel filtration.

Transient expression of cell surface molecules and FACS analysis

To evaluate the binding of the various anti-Fn14 antibodies to different Fn14 variants and to analyze the agonistic activity of anti-Fn14s bound to FcγRs we used co-cultures with HT1080 cells, HEK293 cells were initially electroporated with expression plasmids encoding the protein of interest (Fn14 variants, FcγRs) to achieve transient expression. For this purpose, 40 × 10^6 cells were resuspended in 1 ml RPMI 1640 medium with 10% FCS and supplemented with 30 μg of the appropriate expression construct (in TE buffer, maximum volume 50 μl). The cell/plasmid mixture was transferred in a 4 mm cuvette and electroporated with 250 V and 1800 μF maximum resistance using an EasyJet Plus electroporator (PeqLab). After electroporation, cells were recovered in a 15 cm tissue culture with RPMI 1640 medium containing 10% FCS. On the first or second day after that, cells were harvested and controlled for cell surface expression of the Fn14 variants or the various FcγRs using standard FACS protocols and a FACS-Calibur (BD).

Analysis of IL8 induction

HT1080 cells (2 × 10^4 / well, 96-well tissue culture plates) were seeded in medium containing 10% FCS. The next day, medium was exchanged to minimize the effect of constitutively produced IL8. Cells were treated in triplicates as indicated with the various anti-Fn14 antibodies, anti-Flag mAb M2 (0.5 mg/ml) oligomerized Flag-TWEAK and protein G. After overnight incubation, supernatants were analyzed for IL8 by ELISA. In the co-culture experiments, HEK293 cells transiently expressing FcγRs were cultivated overnight in 96-well tissue culture plates (2 × 10^4 cells/well) and then supplemented with freshly harvested target cells (HT1080 cells, 2 × 10^4 cells/well). Thirty minutes later, the co-cultures were stimulated with the anti-Fn14 antibodies for an additional day and finally IL8 production was again analyzed by ELISA.

Analysis of cellular viability

To evaluate Fn14-triggered cell death, SKOV3 cells were seeded in 96-well tissue plates (1.5 × 10^4 cells/well) and challenged the next day for another day with the indicated combinations of the various anti-Fn14 antibodies, Flag-TWEAK, anti-Flag mAb M2, and protein G. To analyze Fn14-mediated enhancement of TNF-induced cell death, HT29 cells were seeded in 96-well tissue plates (1.5 × 10^4 cells/well) and challenged the next day as indicated with the anti-Fn14 antibodies, Flag-TWEAK, and protein G. One day later, cells were finally challenged for 18 h with 50 ng/ml TNF. In both types of experiments, cellular viability was determined by crystal violet staining.

Activation of the alternative NFκB pathway

HT29 cells (1 × 10^4) were seeded in 6-well plates and treated the next day with Flag-TWEAK or anti-Fn14 antibodies as indicated. To prepare total cell lysates, cells were washed twice with phosphate buffered saline (PBS), harvested in ice-cold PBS with a rubber policeman, resuspended in 4 × Laemmli sample buffer supplemented with a phosphatase inhibitor cocktail II (Sigma) and sonicated for 15 s. Lysates were then boiled for 5 min at 96 °C and subjected to separation by SDS-PAGE. Proteins were transferred to nitrocellulose membranes by standard semi-dry blotting and analyzed by western blotting for the processing of p100 to p52 and accumulation of NIK by the use of appropriate primary antibodies, horseradish peroxidase-conjugated secondary antibodies (Dako) and the enhanced chemiluminescence (ECL) western blotting detection reagents, and analysis system (Amersham Biosciences).
Evaluation of ADCC

ADCC activity of antibodies was determined by the standard 
$^{51}$Cr-release assay. Human PBMCs purified from heparinized whole blood by Ficoll separation were used as effector cells. The target cells used were 786-O cells and the ratio of effector to target cells was 20:1. MAbs were tested for ADCC activity at the indicated concentrations. Lysis was determined after 2 h and percentage of lysis was calculated using the equation: % lysis = 100 × (sample CPM - spontaneous release CPM): (maximum release CPM - spontaneous release CPM). Maximum release was obtained by triton X100 lysis.

Animal studies

BALB/c and nude mice (NMRI-Fox1nu/Fox1nu) were obtained from Charles River. All animal experiments were approved by the Regierung von Unterfranken and complied with the German animal protection law.

In the RENCA model, 16 mice per experimental group were analyzed in two independent series of 8 mice per group. RENCA cells were harvested with trypsin, washed two times with PBS, adjusted to a density of 0.5 × 10^6 cells/ml in PBS and then used to inject 1 × 10^5 cells in the tail vein. Starting the next day, mice were treated intraperitoneally (i.p.) two times per week with 100 µg/ml PBS solutions of 18D1-enhanced, 18D1-dead or golimumab (human TNF-specific IgG1). 26–28 d after tumor cell inoculation mice were sacrificed and analyzed for the presence of metastases. One mouse in the 18D1-dead cohort died 3 d after tumor cell inoculation for unknown reasons and was thus not included in the experiment. Animals (NMRI-Fox1nu/Fox1nu nude mice) were anesthetized, a median laparotomy was performed and after mobilization of the duodenum to liberate the portal vein, luciferase expressing HCT116 cells were injected into the portal vein in 200 µl PBS. After repositioning of the intestine the abdominal wall was closed. Four days later, treatment with 18D1-enhanced and 18D1-dead was started and performed as in “A.” The extent of metastases growth was monitored by bioluminescence imaging.

Figure 5. Anti-metastatic activities of 18D1-enhanced and 18D1-dead. (A) Cells were treated in triplicates for ~36 h with the indicated mixtures of IFNγ (20 ng/ml), Flag-TWEAK (200 ng/ml), anti-Flag mAb M2 (1 µg/ml), and 4G5 (1 µg/ml). Cellular viability was then determined using the MTT assay and normalized to untreated cells. Flag-TWEAK oligomerized with M2 served to mimic the activity of membrane TWEAK. (B) BALB/c mice were intravenously challenged with 10^6 RENCA cells. Starting one day after tumor cell challenge, mice were treated twice per week for two weeks with 100 µg (200 µl PBS, i.p.) of 18D1-dead and 18D1-enhanced or as a negative control with 100 µg of golimumab, a human-TNF specific IgG1 antibody. 26–28 d after tumor cell inoculation mice were sacrificed and analyzed for the presence of metastases. One mouse in the 18D1-dead cohort died 3 d after tumor cell inoculation for unknown reasons and was thus not included in the experiment. (C) Animals (NMRI-Fox1nu/Fox1nu nude mice) were anesthetized, a median laparotomy was performed and after mobilization of the duodenum to liberate the portal vein, luciferase expressing HCT116 cells were injected into the portal vein in 200 µl PBS. After repositioning of the intestine the abdominal wall was closed. Four days later, treatment with 18D1-enhanced and 18D1-dead was started and performed as in “A.” The extent of metastases growth was monitored by bioluminescence imaging. (D) 10^7 MOPC-315.BMP2.FUGLW cells were intravenously applied to Balb/c mice. The next day, treatment (4 weeks, three i.p. injections per week, 100 µg antibody) with 18D1-dead and 18D1-enhanced was started. Tumor/metastases growth was again monitored with bioluminescence imaging after the indicated times. One mouse in the 18D1-enhanced cohort was sacrificed due to high tumor load on day 30 and one mouse in the 18D1-cohort was sacrificed on day 21.
green fluorescent protein (eGFP) were introduced into the HCT116-luc load, mice were anesthetized with 100 µg 18D1-enhanced, 18D1-dead or PBS. To determine the HCT116-luc load, mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine i.p. supplemented with 180 mg/kg D-luciferin for bioluminescence imaging. App. Ten min later, bioluminescence was recorded (NightOWL in vivo imaging system, Berthold Technologies, Bad Wildbad, Germany). Twelve mice per experimental group were analyzed in two independent series of 6 mice per group.

The luciferase expressing MOPC-315.BMP2.FUGLW cells have been established as follows. Firefly luciferase and enhanced green fluorescent protein (eGFP) were introduced into the MOPC-315 (ATCC, Manassa, VA, USA) plasmacytoma cell line for stable expression by lentiviral transduction. Subcloning of bright cells was accomplished with fluorescence-activated cell sorting and subsequent in vivo passaging of MOPC-315 eGFPbright plasmacytoma cells twice in syngeneic Balb/C mice by intravenous injection and subsequent re-isolation from the bone marrow compartment. This new MOPC-315 variant, emits 165 photons/cell/sec and thus is approximately 17-fold brighter than a previously described luciferase expressing MOPC-315 variant.27 106 MOPC-315.BMP2.FUGLW were resuspended in PBS and intravenously injected in the tail vein of BALB/c (H-2d) mice. Mice were treated with 100 µg 18D1-enhanced, 18D1-dead or PBS i.p. three times per week starting one day after tumor cell inoculation and were evaluated by in vivo bioluminescence at the indicated time points. For in vivo bioluminescence analysis, mice were anesthetized (100 mg/kg ketamine, 10 mg/kg xylazine in PBS, i.p.). Luciferin (150 mg/kg) was co-injected and bioluminescence was recorded 10 min later (IVIS Spectrum, Caliper Life Science).

**Disclosure of Potential Conflicts of Interest**

KS and MM are employees of the company arGEN-X BVBA. HW is a consultant of arGEN-X BVBA.

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**Table 1. Homology of variable domains of 18D1, 5B6, and 4G5 to human germ line**

|        | VH identity to human germline (%) | VH homology to human germline (%) | VL identity to human germline (%) | VL homology to human germline (%) |
|--------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| 18D1   | 95.4                             | 97.7                             | 83.5                             | 87.3                             |
| 5B6    | 92                               | 95.4                             | 88.6                             | 88.6                             |
| 4G5    | 94.3                             | 96.6                             | 93.8                             | 83.8                             |

|        | VH identity to human germline (%) | VH homology to human germline (%) | VL identity to human germline (%) | VL homology to human germline (%) |
|--------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| 18D1   | 95.4                             | 97.7                             | 83.5                             | 87.3                             |
| 5B6    | 92                               | 95.4                             | 88.6                             | 88.6                             |
| 4G5    | 94.3                             | 96.6                             | 93.8                             | 83.8                             |
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