Blocking TWEAK-Fn14 interaction inhibits hematopoietic stem cell transplantation-induced intestinal cell death and reduces GvHD

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Running title: Blocking TWEAK-Fn14 interactions in GvHD

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Key points

- Fn14 activation is involved in intestinal apoptosis after allo-HCT and contributes to gastrointestinal GvHD

- Fn14 blockade with an ADCC-defective human IgG1 antibody reduces GvHD severity without modulating GvL responses

Abstract

Inhibition of the TWEAK/Fn14 system reduces intestinal cell death and disease development in several models of colitis. In view of the crucial role of TNF and intestinal cell death in graft-versus-host-disease (GvHD) and the ability of TWEAK to enhance TNF-induced cell death, we tested here the therapeutic potential of Fn14 blockade on allogeneic hematopoietic cell transplantation (allo-HCT) induced intestinal GvHD. A Fn14-specific blocking human IgG1 antibody variant with compromised antibody-dependent cellular cytotoxicity (ADCC) activity strongly inhibited the severity of murine allo-HCT induced GvHD. Treatment of the allo-HCT recipients with this mAb reduced cell death of gastrointestinal cells but neither affected organ infiltration by donor T-cells nor cytokine production. Fn14 blockade also inhibited intestinal cell death in mice challenged with TNF. This suggests that the protective effect of Fn14 blockade in allo-HCT is based on the protection of intestinal cells from TNF-induced apoptosis and not due to immune suppression. Importantly, Fn14 blockade showed no negative effect on graft-versus-lymphoma (GvL) activity. Thus, ADCC-defective Fn14-blocking antibodies are not only possible novel GvL effect-sparing therapeutics for the treatment of GvHD but might also be...
useful for the treatment of other inflammatory bowel diseases where TNF-induced cell death is of relevance.

**Introduction**

Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK, TNFSF12) is a typical member of the TNF ligand family and its receptor fibroblast growth factor-inducible 14 (Fn14, TNFRSF12a) belongs to the TNF receptor associated factor (TRAF)-interacting subgroup of the TNF receptor family.\(^1\) Like most other ligands of the TNF family, TWEAK is a single-spanning transmembrane protein with an extracellular C-terminal TNF homology domain (THD) followed by a stalk region connecting the THD with the transmembrane domain and the cytoplasmic N-terminal part of the molecule. Not unusual for a TNF ligand, the stalk region of TWEAK is subject to proteolytic processing and thus allows the generation of a soluble form of TWEAK. At the mRNA level TWEAK expression has been documented for a variety of cell lines and in many tissues. Cell surface exposed membrane bound TWEAK, however, has so far only been reported for monocytes, macrophages, dendritic cells, natural killer cells and a few cancer cell lines. Fn14 is strongly expressed in all tissue during development but shows a differentiated expression pattern in the adult organism reaching from high expression in heart and ovary over weak expression in brain and skeletal muscle to lack of detectable expression in the spleen.\(^3\) Particularly, in accordance with its identification as a FGF-inducible protein, Fn14 was found to be strongly induced by various growth factors and cytokines\(^4\)\(^-\)\(^8\), thus in situations of tissue damage.\(^9\)\(^,\)\(^10\) The TWEAK/Fn14 system triggers a diverse range of cellular effects including the stimulation of angiogenesis, proliferation, cell differentiation and cell migration but also the activation of proinflammatory gene transcription programs and in rare cases apoptosis. The range
of activities of the TWEAK/Fn14 system and the tissue damage/injury-associated expression pattern of Fn14 argue for a role of TWEAK and Fn14 in wound healing, tissue repair, regeneration and maintenance of tissue homeostasis. In line with this, it has been found that TWEAK and Fn14 are required for the regenerative responses occurring after muscle injury, partial hepatectomy and partial pancreatectomy. In the case of exaggerated or chronic activation, however, the TWEAK/Fn14 system may also contribute to tissue injury. Indeed, in most disease models investigated so far, genetic or pharmacological inactivation of the TWEAK/Fn14 system showed a beneficial effect.

Allogeneic hematopoietic cell transplantation (allo-HCT) is often the only curative treatment option for a number of malignant and non-malignant diseases of the hematopoietic system. With respect to the treatment of leukemia by allo-HCT a crucial issue is the so called graft-versus-leukemia/lymphoma (GvL) effect, a donor T cell and NK cell mediated immune response against residual malignant cells in the recipient that have survived previous treatments with chemo- and/or radiotherapy. However, the GvL activity is closely linked to immune reactions of donor cells against normal non-transformed host cells leading to graft-versus-host-disease (GvHD), one of the main reasons of mortality after allo-HCT. Acute GvHD mainly affects the gastrointestinal tract, liver, skin.

Inhibition of the TWEAK/Fn14 signaling showed a protective effect in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced, IL-10 deficiency-induced, and γ-irradiation-induced colitis. Thus, we evaluated whether blockade of Fn14 would interfere with intestinal GvHD following allo-HCT. We found that a recombinant Fn14-specific blocking human IgG1 antibody strongly reduced the severity of allo-HCT induced GvHD in mice without interfering with GvL activity. While an antibody variant with compromised FcγR binding was effective, an antibody variant
with enhanced antibody-dependent cellular cytotoxicity (ADCC) activity failed to show any protective effect. This suggests that the therapeutic effect is indeed due to inhibition of Fn14 signaling and not related to ADCC-mediated depletion of Fn14-expressing cells or activation of Fn14 by Fcγ-receptor (FcγR)-bound antibody.

**Methods**

**Antibodies and Animals**

The anti-Fn14 hIgG1 variants 18D1-dead and 18D1-enhanced have been described in detail elsewhere. Rituximab, a therapeutic human IgG1 antibody recognizing human but not murine CD20, was from Hoffmann La-Roche (Basel, Switzerland). Balb/c and C57Bl/6 (B6) mice were purchased from Charles River (Sulzfeld, Germany). Firefly luciferase-transgenic B6.L2G85.CD90.1 mice were described in detail elsewhere. For all experiments female mice between 8 and 12 weeks of age were used. Mice were bred within the specified pathogen-free animal facility of the Center for Experimental Molecular Medicine of the University Hospital Würzburg receiving rodent chow and autoclaved drinking water ad libitum. All animal experiments were approved by local authorities (Regierung von Unterfranken, reference number 55.2-2531.01-103/11) and complied with German animal protection law.

**Cells and cell culture**

Bone marrow cells were isolated from B6 mice by flushing femur and tibia bones with phosphate buffered saline (PBS) and filtration of the obtained cell suspension through a 70 µm cell strainer (BD, Heidelberg, Germany). For preparation of T-cells, spleens were directly filtered through a
70 µm cell strainer into erythrocyte lysis buffer (168 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM ethylenediaminetetraacetic acid) and incubated for 2 minutes. Two volumes of PBS were added to the single cell suspension and cells were spun down. Cells were resuspended in PBS, filtered through a new 70 µm cell strainer and were spun down again. Cells were resuspended in PBS and T-cells were enriched using the Dynal Mouse T-cell Negative Isolation Kit (Invitrogen, Darmstadt, Germany) according to the manufacturer’s instructions. IM380 plasmablastic lymphoma (H-2<sup>d</sup>) cells stably expressing firefly luciferase and eGFP (IM380 egfp/luc)<sup>25</sup>, and A20 yfp/luc mouse B-cell lymphoma (H-2<sup>d</sup>) cells stably expressing firefly luciferase and YFP<sup>26</sup> were maintained in RPMI medium supplemented with 10% fetal calf serum and 1% antibiotics (penicillin/streptomycin) and passaged twice weekly. Both cell lines are syngeneic to Balb/c mice.

**Allogeneic hematopoietic cell transplantation and graft-versus-host disease/graft-versus-lymphoma model**

Female Balb/c mice (H-2<sup>d</sup>), 8-12 weeks of age, were lethally irradiated with a dose of 8 Gy using a Faxitron CP-160 X-ray irradiation system (Faxitron X-Ray, Lincolnshire, IL, USA). To induce GvHD, conditioned mice were then injected with 5x10<sup>6</sup> allogeneic bone marrow cells isolated from B6 donor mice (H-2<sup>b</sup>) and 1x10<sup>6</sup> enriched T-cells from B6 or firefly luciferase-transgenic B6.L2G85.CD90.1 mice into the retro-orbital plexus. For GvL experiments with A20 yfp/luc cells, 1x10<sup>5</sup> cells in 100 µl PBS were injected into the lateral tail vein of host mice following irradiation and transfer of bone marrow cells and T-cells. For GvL experiments with IM380 egfp/luc cells, 1x10<sup>5</sup> cells were injected into the lateral tail vein of host mice six days prior to allo-HCT. Drinking water of mice receiving allo-HCT was supplemented with antibiotic
(Baytril, Bayer, Leverkusen, Germany) for one week to prevent infections following myeloablative irradiation. To target Fn14, mice were treated daily for a week with 100 µg of 18D1-enhanced or 18D1-dead (i.p. 100 µl PBS) starting one day after allo-HCT. Rituximab (anti-human CD20 IgG1) served as a negative control. Mice were assessed daily for weight loss and clinical scoring of GvHD symptoms (adapted from ref. 26) for the first 10 days after allo-HCT and then every other day until end of the experiment. To avoid overestimation of late effects, i.e. when only few mice are still alive and have comparably low scores, the clinical scoring data were arranged in the way that mice that died from GvHD or that were euthanized due to ethical reasons (score > 8, weight loss > 20 %) were included in the statistical evaluation with their last score until the last mouse of the group had died. Thus, the fact that a graph ends earlier than the whole experiment indicates that all mice were severely affected so that they had to be euthanized.

**Bioluminescence imaging**

For *in vivo* bioluminescence imaging mice were i.p. injected with 80 mg/kg esketamine hydrochloride (Pfizer, Berlin, Germany) and 16 mg/kg xylazine (cp Pharma, Burgdorf, Germany) for anesthetization together with 300 mg/kg of the luciferase substrate D-luciferin (Biosynth, Staad, Switzerland). Bioluminescence signals were recorded 10 min later from ventral and lateral views with a maximum exposure time of five minutes per picture using an IVIS Spectrum imaging system (Caliper Life Sciences, Mainz, Germany). Pictures were evaluated using Living Image 4.0 software (Caliper Life Sciences). For *ex vivo* imaging mice were injected with 300 mg/kg D-luciferin and euthanized 10 minutes later to prepare internal organs, which were immediately subjected to *ex vivo* bioluminescence imaging.
**Immunohistochemistry and analysis of cytokine expression**

Tissue samples were embedded in Tissue Tek OCT (Sakura Finetek, Staufen, Germany) or stored in 4% PFA for standard histopathological analysis. Tissues were scored (0-4 depending on severity) for the following endpoints: small bowel: apoptosis of crypt cells, inflammation; large bowel: apoptosis of crypt cells, inflammation; liver: bile duct injury, vascular injury, hepatocellular damage, portal inflammation.\(^{27}\) For the evaluation of cytokine expression, serum samples were analysed by help of the BD Cytometric Bead Array Kit (BD Biosciences Pharmingen, Heidelberg) according to the manufacturer’s protocol. Data were analysed with the FCAP Array v2.0 software.

**TNF-induced in vivo apoptosis of intestinal cells**

Twenty week-old male C57Bl/6 (n = 24) mice were randomized into four groups and injected intraperitoneally with 18D1-dead (200 µg in PBS), recombinant murine TNF (10 µg in PBS), a mixture of both, or with saline. After 6 h mice were euthanized and samples of the small intestinal tissue were formalin-fixed and paraffin-embedded at the time of collection. After deparaffinization, rehydration, antigen-retrieval and peroxidase blocking using standard protocols, apoptotic cell death in tissue sections was detected using an anti-cleaved caspase-3 antibody (Asp175, Cell Signaling Technology, Danvers, MA) and an anti-cleaved lamin A antibody (small subunit, Cell Signaling Technology) as described elsewhere.\(^{28}\) Briefly, after incubation with the primary antibodies, sections were washed with TBS and then incubated with Biotin-SP-conjugated AffiniPure, goat anti-rabbit IgG, (1:200, Jackson Immuno Research, Suffolk, UK) for 45 min. Unbound antibodies were removed by two washes with TBS and bound immunocomplexes were visualized using the Vectastain ABC Kit and the diaminobenzidine-
based ImmPACT DAB SK-4105 staining kit (both Vector Laboratories, Peterborough, UK). Sections were counterstained with hematoxylin and evaluated and photographed with the PALM MicroBeam microscope (Carl Zeiss, Göttingen, Germany). The number of apoptotic cells per 100 crypts was determined based on at least 600 crypts.

RNA isolation and qPCR analysis

Small and large bowel were isolated from euthanized mice and snap frozen in liquid nitrogen for isolation of total RNA with the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the protocol of the supplier. Total RNA (1 µg) was subjected to reverse transcription using the QuantiTect Reverse Transcription kit (Qiagen) to synthesize cDNA. Two percent of the reverse transcription reactions were subsequently used as template for quantitative real time PCR with the BioRad CT1000 thermal cycler equipped with the CFX96 Real Time System (BioRad, Munich, Germany) and the QuantiTect SYBR Green PCR Kit (Qiagen). Reactions were run using the following program: I. 95°C, hot start; II. 40 cycles of 15 s at 95°C followed by 30 s at 52 °C and 30 s at 72°C. In all experiments a reaction without cDNA was included as a negative control. For amplification of murine TNF and β-actin cDNA, primer pairs from Qiagen (TNF: QT00104006; ActB: QT00095242) were used. The relative expression (rel. exp.) of the TNF mRNA was calculated using the CT-values of the TNF (CT(TNF)) and β-actin (CT(βact)) PCR reactions and the formula: rel. exp. = \(2^{(CT(TNF) - CT(βact))}\).
Cleaved PARP1 Immunofluorescence microscopy

Sections of 3 µm thickness were cut from cryo-embedded tissues on a Leica CM1900 cryostat (Leica Microsystems, Wetzlar, Germany). Slides were air-dried, fixed with acetone (room temperature, 7 min) and washed and blocked with 2% FBS in PBS for 15 minutes. Slides were incubated with the rabbit monoclonal anti-cleaved PARP antibody E51 (ab32064, Abcam, Cambridge, UK) for 1 hour at room temperature and unbound antibodies were removed by three washes with PBS. Bound antibodies were then detected with Alexa Fluor 647 goat anti-rabbit IgG (Invitrogen). After additional three washes with PBS, slides were counterstained with DAPI and mounted with mounting medium (Vector Laboratories). Images were obtained with a Zeiss Imager.Z1m fluorescence microscope and evaluated using the Zeiss AxioVision software (Carl Zeiss).

Immunohistochemistry

Five formalin fixed and paraffin-embedded (FFPE) human colonic biopsies showing moderate to strong alterations associated with GvHD were selected from the files of the Institute of Pathology, University of Würzburg. Five normal colonic biopsies served as controls. Ethics approval was obtained from the Ethics Committee of the Medical Faculty of the University of Würzburg. After antigen retrieval with target retrieval solution, pH6.1 (Dako, Hamburg, Germany) slides were stained using an anti-Fn14 antibody (sc-56250, Santa Cruz, Heidelberg, Germany) at a dilution of 1:100. Representative images were taken using a Nikon Eclipse E600 microscope equipped with a Nikon DS-Fi1 camera.
Statistics

All data are shown as mean ± standard error of mean (S.E.M.) and represent combined data from at least two independent experiments unless noted otherwise. Figures were prepared using GraphPad Prism 5 software (La Jolla, CA, USA) and Adobe Photoshop 7 (San Jose, CA, USA) or CorelDraw X4 (Corel Corporation, Menlo Park, CA, USA). Data were tested for normality using the Kolmorrnov-Smirnov test or the Shapiro-Wilk test, where appropriate. Normally distributed groups (Figures 1C, 2, 3 and 4B) were compared by two-tailed student’s t-test, not normally distributed groups (Figures 1D and 4A) were compared by Mann-Whitney test. Survival data were analyzed with the Log-rank (Mantel-Cox) test. Data reaching statistical significance are indicated as: * p ≤ 0.05, ** p ≤ 0.01.

Results

Fn14 blockade reduces GvHD severity and prolongs survival after allo-HCT

The histopathology of spontaneous colitis of IL10 knockout mice and acute TNBS-induced colitis, two disease models where the inhibition of the TWEAK/Fn14-system has a protective effect, resembles the clinical manifestations and histological changes observed in intestinal GvHD following allo-HCT. Therefore, we evaluated normal colonic mucosa biopsies and mucosa biopsies derived of patients showing moderate to severe GvHD-associated changes for Fn14 expression. In the lamina propria of all biopsies, we observed numerous Fn14-positive immune cells (Figure 1A). However, the situation was different in intestinal epithelial cells. While epithelial cells remained Fn14-negative in the normal mucosa, pre-apoptotic and apoptotic cryptic glands in the GvHD biopsies stained distinctly positive for Fn14 (Figure 1A,
Supplemental Figure 1). Together this opened the possibility that Fn14 blockade could be protective in inflammatory scenarios with cell death of intestinal epithelial cells. Therefore, we evaluated the effect of the blocking human/murine Fn14-specific antibody 18D1 on GvHD in a major MHC mismatch model in which lethally irradiated Balb/c recipient mice were reconstituted with B6 bone marrow cells and firefly luciferase-transgenic B6 T-cells. Starting at the first day post transplantation mice were treated with an ADCC-defective (18D1-dead) and an ADCC-enhanced (18D1-enhanced) variant of 18D1 as well as with an irrelevant hIgG1 control antibody. Treatment of mice with the 18D1-dead antibody resulted in significantly prolonged survival compared to transplanted mice treated with the control hIgG1 while 18D1-enhanced had no effect (median survival: hIgG: 8 days, 18D1-enhanced: 8 days, 18D1-dead > 30 days; Figure 1B). Mice receiving 18D1-dead after allo-HCT initially lost as much weight as did control hIgG1-treated mice, but recovered from allo-HCT by day 9 after transplantation (Figure 1C). Prolonged survival of mice treated with 18D1-dead came along with a stabilization of the clinical score (Figure 1D).

Fn14 blockade reduces gastrointestinal organ damage in GvHD mice

In vivo-bioluminescence imaging showed no difference in global donor T-cell expansion after allo-HCT between mice treated with 18D1-dead and hIgG1 (Figure 2A). To assess donor T-cell proliferation and expansion patterns in more detail, we performed ex vivo imaging on day 6 after allo-HCT. Again there was no evidence that 18D1-dead modified organ infiltration by alloreactive luciferase-transgenic T-cells (Figure 2B). Quantitative PCR analysis revealed a strong increase in the expression of TNF in bowel biopsies of mice with GvHD compared to healthy mice and this increase in TNF expression was not affected by treatment with 18D1-dead.
To evaluate further whether treatment with 18D1-dead affects systemic cytokine levels, we performed cytometric bead array analysis of serum samples. Serum levels of IL-6, IFN-γ and TNF were significantly increased after allo-HCT while the level of IL-2 was reduced but there were no significant differences between mice treated with 18D1-dead and hIgG1 (Figure 2D). Collectively, these data suggest that the protective effect of 18D1-dead in GvHD is not related to the inhibition of potential proinflammatory Fn14 activities.

**Fn14 blockade inhibits intestinal apoptosis after allo-HCT**

Apoptosis induction in intestinal epithelial cells by γ-irradiation is reduced in TWEAK- and Fn14-knockout mice. It has been furthermore observed in an intestinal explant system that apoptosis induction by exogenous TNF is diminished in primary cultures derived of Fn14-knockout mice. Indeed, TNF is considered to be important in GvHD not only due to its stimulatory effects on T-cells but also as an effector molecule that contributes to apoptosis induction in GvHD target tissues. Moreover, TNF is generally a prominent inducer of cell death in the intestinal epithelium. Against the background of the increased expression of TNF in the gut after allo-HCT (Figure 2C) and the established strong ability of the TWEAK/Fn14-system to enhance TNFR1-induced cell death, we addressed the possibility that 18D1-dead affects intestinal apoptosis after allo-HCT. Immunohistochemical evaluation of the gastrointestinal tract of control hIgG1 and 18D1-dead treated mice revealed a significant reduction in the number of cleaved PARP1-positive cells within the small bowel (Figure 3A). This suggests that the protective effect of 18D1-dead on GvHD after allo-HCT is based on the inhibition of the apoptotic crosstalk of TWEAK and TNF in intestinal epithelial cells. In further accordance with this idea, 18D1-dead also showed a protective effect on intestinal cell apoptosis
after exogenous administration of TNF in Balb/c \textit{(Figure 3B)} as well as in B6 mice \textit{(Supplemental Figure 2)}.

\textbf{Fn14 blockade inhibits GvHD after allo-HCT while maintaining GvL effects}

To assess whether blocking of Fn14 with 18D1-dead affects the capacity of the transplanted allogeneic T-cells to control tumor cells, we performed GvL experiments with two different B-cell lymphoma cell lines, A20 and IM380. In the case of the A20 model, tumor cells were co-transplanted together with the bone marrow graft and the allogeneic T-cells, while in the genetically induced IM380 model, mice with pre-established tumors were subjected to allo-HCT. In both models, the tumors initially (4-5 days) developed similarly after allo-HCT irrespective whether the mice were transplanted with bone marrow cells alone or with bone marrow cells supplemented with allogeneic T-cells \textit{(Figure 4A)}. Mice transplanted with allogeneic T-cell supplemented bone marrow cells subsequently rejected the tumors while the bone marrow cell-transplanted mice did not \textit{(Figure 4A)}. Treatment with 18D1-dead showed no effect on tumor rejection \textit{(Figure 4A)} indicating that Fn14-blockade does not interfere with GvL activity. Moreover, 18D1-dead treatment again significantly prolonged survival and prevented deadly weight loss of mice that showed GvL and developed GvHD \textit{(Figure 4A,B)}.

\textbf{Discussion}

By activation of its receptor Fn14, TWEAK stimulates the production of proinflammatory cytokines and chemokines in a variety of cell types\textsuperscript{39-42} TWEAK also acts as a sensitizer for TNF-induced cell death\textsuperscript{36-38} Noteworthy, in murine models of colitis, TWEAK crucially
contributes to disease progression by inducing colon epithelial cells to secrete chemokines causing immune cell infiltration, as well as TNF-dependent cell death of intestinal epithelial cells. As inflammation and intestinal cell death-induction propagate intestinal GvHD pathophysiology, we asked whether the TWEAK/Fn14-system may serve as a useful therapeutic target in this disease. To address this, we treated Balb/c mice that had been transplanted with T-cells and bone marrow cells derived from allogeneic B6 mice with the Fn14-specific antibody 18D1. This llama-derived antibody efficiently blocks TWEAK binding to human and murine Fn14. When expressed as a human IgG1, 18D1 elicits a strong inhibitory effect on soluble and membrane TWEAK-induced Fn14-signaling. However, like other Fn14-specific hIgG1 antibodies, 18D1 acquires a high agonistic potential upon oligomerization and FcγR-binding and has then similar signaling activities as membrane TWEAK. To distinguish whether a potential effect of 18D1 depends on FcγR binding or not, we evaluated two variants of 18D1 with mutated Fc domains. One conferred an enhanced ability to bind to FcγRs and to trigger ADCC. The other one displayed a reduced FcγR binding ability and thus with impaired ADCC activity. The ADCC-enhanced variant of 18D1 showed no protective effect on intestinal GvHD after allo-HCT while its ADCC-defective counterpart 18D1-dead strongly diminished gastrointestinal GvHD (Figure 1). This observation suggests that antibody immune effector functions of 18D1 may limit protective effects related to Fn14 blockade and we thus focused in the following experiments on evaluation of the 18D1-dead variant. The disease promoting effect of Fn14 in murine colitis models has been attributed to an enhancement of TNF-dependent apoptosis in intestinal epithelial cells and disturbance of the barrier function of the gut epithelium. The observation that the inhibition of intestinal tissue damage by 18D1-dead is accompanied by reduced numbers of apoptotic cells and reduced caspase processing in the
intestine suggests that the therapeutic effect of 18D1-dead relies on the inhibition of TNF/TWEAK-induced cell death, too. Indeed, we found no evidence for an immune-suppressive effect of 18D1-dead in intestinal GvHD indicating that early events after allo-HCT are not influenced by Fn14 blockade (Figure 2). The observation that 18D1-dead showed no major effect on the inflammatory aspects of GvHD may be due to the fact that TWEAK mainly acts on parenchymal cells rather than adaptive immune cells, which do not express Fn14 and that in the acute GvHD model used here, the main cytokine-producing cells are alloreactive T-cells.\(^\text{10,44,45}\) Thus, blocking of Fn14 appears to act later in GvHD pathophysiology by inhibiting apoptotic tissue damage induced by infiltrating donor T cells. This corresponds to the fact that the protective effect of 18D1-dead becomes not apparent in the first week after allo-HCT (Figure 1). The finding that Fn14 blockade does not compromise the activity of donor derived T-cells also explains the unaffected GvL activity observed in 18D1-dead treated mice (Figure 4). This is of particular relevance as immune-suppressive treatment regimes typically not only ameliorate GvHD but also interfere with the therapeutically desired GvL effect. It is worth mentioning that TNF and its receptors are at least in two different ways of crucial relevance for donor T-cell activity and thus for GvHD and GvL. First, donor T-cell expressed TNF acts as an effector molecule in GVHD and GvL. Furthermore, both TNFR1 and TNFR2 are required by donor T-cells in GvHD models for \textit{in vivo} generation of a proper allo-specific cytotoxic T-cell response.\(^\text{46-48}\) Thus, in contrast to TNF blockers, which potentially interfere with GvHD and GvL and are used in clinical practice as an experimental mean to treat GvHD (for review see ref. 49), inhibitors of the TWEAK/Fn14-system might elicit a more selective effect on GvHD only.

The fact that the ADCC-enhanced variant of 18D1 lacks therapeutic efficacy in GvHD after allo-HCT suggests that the ability to inhibit stimulation of Fn14 by endogenous TWEAK is
indeed the main mode of action of 18D1 in this model. Although treatment with 18D1-enhanced 
would also prevent Fn14 stimulation by endogenous TWEAK, one has to consider that this 
protective effect could be counteracted by FcγR binding. This could result in a change from 
TNF/TWEAK-mediated killing of intestinal epithelial cells to ADCC-mediated cell lysis. In the 
case of insensitivity of intestinal epithelial cells for ADCC the FcγR binding of 18D1-enhanced 
should unleash the latently present agonistic activity of the 18D1 antibody. Thus, stimulation of 
Fn14 by endogenous TWEAK would only be substituted by stimulation with FcγR-bound 18D1 
with no major effect on the TNF/TWEAK crosstalk. In line with this idea, it has been recently 
shown that human IgG1 antibodies targeting CD40 or the TRAIL death receptors elicit strong 
FcγR-dependent agonistic activity in mice.\textsuperscript{50-53}

Noteworthy, blockade of Fn14 might not only reduce intestinal manifestations of acute 
GvHD as shown in this study but might also reduce disease severity in other organs. So, it has 
been reported that Fn14 knockout mice and mice treated with a TWEAK-neutralizing antibody 
are protected in a model of chronic graft-versus-host induced lupus erythematosus.\textsuperscript{54} In this 
model activated donor CD4 T-cells deliver T-cell help to recipient B-cells and so trigger the 
production of autoantibodies and lupus disease symptoms.

TNF-induced cell death of intestinal epithelial cells has been identified as a pivotal step in 
the pathogenesis of various inflammatory diseases of the bowel. Typically, the TNF-dependent 
cell death-enhancing TWEAK/Fn14-system is highly active in stressed/injured tissue. Therefore, 
it is tempting to speculate that TWEAK and Fn14 also contribute to other intestinal diseases 
where TNF-induced cell death is of relevance. Thus, ADCC-defective Fn14-blocking antibodies 
are not only potential novel GvL effect-sparing therapeutics for the treatment and prevention of 
GvHD but might also useful for the treatment of other inflammatory bowel diseases.
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Contribution

H.W., A.B., M.C. and T.B. designed the experiments. H.W., A.B., M.C., T.B., H.E. and A.R. wrote and edited the manuscript. M.C., A.B., M.B., S.K., C.A.B., M.R., K.M., S.S performed and evaluated the GvHD-related animal experiments. T.G. and T.B. conducted the TNF-related animal experiment. V.S. produced and purified the Fn14-specific antibody variants and the recombinant TNF. D.S. and A.S. performed the qPCR analysis. A.M. and A.R. executed the immunohistochemistry evaluation of the GvHD biopsies.

Conflict-of-interest disclosure

H.W. is a consultant of Argen-X BVBA, the developer of the Fn14-specific antibodies used in this study.

Data and materials availability

The 18D1-dead and 18D1-enhanced antibodies must be obtained through an MTA.
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Figure legends

Figure 1: An ADCC-defective variant of the anti-Fn14 mAb 18D reduces acute GvHD and prolongs survival after allo-HCT. (A) Increased expression of Fn14 in intestinal epithelial cells of GvHD patients. Sections of normal mucosa (left) and mucosa with GvHD associated changes (right) were stained with an anti-Fn14 antibody (ITEM-4) (in brown). Representative results are shown. Original magnification was x200. Fn14-positive immune cells are present in the lamina propria of both sample types, while Fn14-positive epithelial cells are only observed in GvHD samples. (B-D) Balb/c (H-2^d^) mice were myeloablativey irradiated and transplanted with 5x10^6^ B6 (H-2^b^) bone marrow cells and 1x10^6^ enriched B6.L2G85.CD90.1 (H-2^b^) T-cells. Starting one day post transplantation mice were treated daily with 100 µg of 18D1-dead, 18D1-enhanced or an irrelevant hIgG1 control antibody. Shown are combined data from three independent experiments (18D1-dead: n = 20, 18D1-enhanced: n = 10, hIgG1: n = 20). To control the efficacy of the myeloablative conditioning (irradiation only) and bone marrow engraftment (bone marrow control), mice were irradiated without transplantation or were irradiated and only transplanted with B6 (H-2^b^) bone marrow cells. (B) Survival of allo-HCT recipients. (C) Mice were weighed at the indicated times after allo-HCT. Weight measurements are shown in percent of initial weight. (D) Mice were assessed for clinical signs of GvHD at the indicated time points. Mean ± S.E.M., * p ≤ 0.05, ** p ≤ 0.01 (hIgG1 vs. 18D1-dead).

Figure 2: Treatment of allo-HCT recipients with the ADCC-defective anti-Fn14 mAb variant 18D1-dead does not affect donor T-cell target organ infiltration and cytokine production. Lethally irradiated Balb/c (H-2^d^) mice were transplanted with 5x10^6^ B6 (H-2^b^) bone
marrow cells alone or together with 1x10^6 enriched B6.L2G85.CD90.1 (H-2^b) T-cells. Mice of the latter group were daily treated with 100 µg of 18D1-dead or an irrelevant hIgG1 control antibody for six days. All mice (n = 5 per group) were then euthanized for *ex vivo* assessment of T-cell expansion, cytokine production and cell death-induction in the gastrointestinal tract. (A) *In vivo* bioluminescence imaging of B6.L2G85.CD90.1 (H-2^b) cells in transplanted antibody-treated mice. Bioluminescence was imaged at indicated time points and light emission of donor T-cells quantified. The upper panel shows the average light emission from ventral view and the lower panel shows images from one representative mouse of each group. (B) Internal organs were analyzed *ex vivo* for donor T-cell-derived bioluminescence activity (lu: lung, cLN: cervical lymph nodes, thy: thymus, hea: heart, ki: kidney, iLN: inguinal lymph nodes, li: liver, sb: small bowel, lb: large bowel, mLN: mesenteric lymph nodes, st: stomach, cae: caecum, spl: spleen). Bioluminescence images of the organs of one representative mouse of each group are shown in the upper panel. The organ-derived averaged emissions are shown in the lower panel. (C) Large bowel biopsies of 18D1-dead and control hIgG1 treated GvHD mice and of untreated control mice were analyzed by qPCR for TNF expression. (D) Concentrations of various cytokines in serum were determined by help of a cytometric bead array. Mean ± S.E.M., * p ≤ 0.05, ** p ≤ 0.01.

**Figure 3: The ADCC-defective anti-Fn14 mAb variant 18D1-dead reduces allo-HCT-triggered and TNF-induced cell death of gastrointestinal cells.** (A) 3 µm intestinal tissue sections from mice shown in figure 2 were analyzed by immunohistochemistry with a cleaved PARP1-specific antibody. Upper panel: graphic evaluation of apoptotic cells per field. Lower panel: representative photomicrographs. (B) Balb/c (H-2^d) were injected with 18D1-dead (200
µg in PBs), murine TNF (10 µg in PBS), a mixture of both or with saline. After 6 h mice were euthanized and small intestinal tissues were analyzed by immunohistochemistry for the presence of apoptotic cells with anti-cleaved caspase-3 (upper panel) and anti-cleaved lamin A-specific antibodies (lower panel). Mean ± S.E.M., * p ≤ 0.05.

**Figure 4: Treatment of allo-HCT recipients with the ADCC-defective anti-Fn14 mAb variant 18D1-dead delays the onset of acute GvHD while maintaining GvL.** The effect of 18D1-dead on GvL activity was investigated in IM380 and A20 B cell lymphoma models. Balb/c (H-2<sup>d</sup>) mice were either injected with 10<sup>5</sup> syngenic, luciferase-transgenic IM380 B cell lymphoma cells i.v. six days before allo-HCT or with 10<sup>5</sup> syngenic, luciferase-transgenic A20 cells along with allogeneic 5x10<sup>6</sup> B6 (H-2<sup>b</sup>) bone marrow cells alone or together with 1x10<sup>6</sup> enriched B6 (H-2<sup>b</sup>) T-cells. Starting one day post allo-HCT mice were treated daily for a week with 100 µg of 18D1-dead or with 100 µg of an irrelevant hIgG1 mAb (n = 5 for each group). (A) *In vivo* bioluminescence imaging of luciferase<sup>+</sup> lymphoma cells of a representative mouse of the indicated groups. Upper panels represent data at eight days after transplantation. (B) Survival analysis of allo-HCT recipients (reight panel). Data from both GvL models are displayed along with data from mice of the myeloablative conditioning control (irradiation only) and the bone marrow engraftment control (bone marrow control). Weight change after allo-HCT in percent of the initial weight (left panel). Statistics of hIgG1 vs. 18D1-dead are indicated: Mean ± S.E.M., * p ≤ 0.05, ** p ≤ 0.01.
Figure 1

A control

GvHD

B

Survival (%)

Days after allo-HCT

C

Relative weight (%)

Days after allo-HCT

D

Clinical score

Days after allo-HCT

- BM control
- irradiation control
- untreated
- hlG1
- 18D1-enhanced
- 18D1-dead

p<0.001

* - **
Figure 3

A. Cleaved PARP1+ (cells/field)

- BM only
- hlgG1
- 18D1-dead

Small bowel

B. Cleaved caspase-3+ (cells/100 crypts)

- 18D1-dead
- TNF
- 18D1-dead + TNF

B. Cleaved lamin A+ (cells/100 crypts)

- BM only
- hlgG1
- 18D1-dead
Figure 4

A

10^8

10^7

10^6

10^5

photons per s

hlgG1 18D1 hlgG1 18D1

BM only BM + T-cells

IM380

p=0.1110

p=0.0079

10^8

10^7

10^6

10^5

photons per s

hlgG1 18D1 hlgG1 18D1

BM only BM + T-cells

A20

B

Days after allo-HCT

Relative weight (%)

0 10 20 30 40 50 60

70 80 90 100 110

Days after allo-HCT

Survival (%)

0 20 40 50 60

Days after luciferase^+ T_con infusion

irradiation control GVHD IM380 hlgG
BM control GVHD IM380 Fn14

GVHD A20 hlgG

GVHD A20 Fn14

* p < 0.05

**
Blocking TWEAK-Fn14 interaction inhibits hematopoietic stem cell transplantation-induced intestinal cell death and reduces GvHD

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