Cell Surface Delivery of TRAIL Strongly Augments the Tumoridical Activity of T Cells

Marco de Bruyn1, Yunwei Wei1,3, Valerie R. Wiersma1, Douwe F. Samplonius1, Harry G. Klip2, Ate G.J. van der Zee2, Baofeng Yang4, Wijnand Helfrich1, and Edwin Bremer1

Abstract

Purpose: Adoptive T-cell therapy generally fails to induce meaningful anticancer responses in patients with solid tumors. Here, we present a novel strategy designed to selectively enhance the tumoridical activity of T cells by targeted delivery of TNF-related apoptosis-inducing ligand (TRAIL) to the T-cell surface.

Experimental Design: We constructed two recombinant fusion proteins, anti-CD3:TRAIL and K12:TRAIL. Tumoridical activity of T cells in the presence of these fusion proteins was assessed in solid tumor cell lines, primary patient-derived malignant cells, and in a murine xenograft model.

Results: When added to T cells, K12:TRAIL and anti-CD3:TRAIL selectively bind to the T-cell surface antigens CD3 and CD7, respectively, leading to cell surface accretion of TRAIL. Subsequently, anti-CD3:TRAIL and K12:TRAIL increased the tumoridical activity of T cells toward cancer cell lines and primary patient-derived malignant cells by more than 500-fold. Furthermore, T-cell surface delivery of TRAIL strongly inhibited tumor growth and increased survival time of xenografted mice more than 6-fold.

Conclusions: Targeted delivery of TRAIL to cell surface antigens of T cells potently enhances the tumoridical activity of T cells. This approach may be generally applicable to enhance the efficacy of adoptive T-cell therapy. Clin Cancer Res; 17(17); 5626–37. ©2011 AACR.

Introduction

While efficacious in certain virus-mediated cancers, adoptive T-cell therapy generally fails to induce meaningful anticancer responses in patients with solid tumors. The cause for this lack of clinical efficacy is multifactorial by nature and includes the intrinsic or acquired resistance of malignant cells to cytotoxic T-cell effector mechanisms such as granzyme/perforin-mediated lysis (1–5) and Fas-mediated killing (6, 7). To overcome or circumvent this potential pitfall of adoptive T-cell therapy, we propose to selectively expand the T-cell armamentarium with additional tumoridical effector molecules, in particular with the TNF-related apoptosis-inducing ligand (TRAIL).

TRAIL is a type II transmembrane protein normally expressed on natural killer (NK) cells, where it is essential for NK cell–mediated tumor immune surveillance (8, 9). In contrast, the expression of TRAIL on resting T cells is typically very low (10, 11). Nevertheless, a slight induction of TRAIL expression on T cells, by ectopic expression of TRAIL or by ex vivo costimulation with anti-CD3 and IFN-α, already potently increases their tumoridical activity (11, 12). Furthermore, T-cell–expressed TRAIL is essential for graft-versus-tumor (GVT) activity during allogeneic hematopoietic cell transplantation (AHCT; ref. 10). Importantly, T-cell–expressed TRAIL does not contribute to deleterious GVHD activity (10). Taken together, these findings suggest that T cells should gain additional tumoridical potential when equipped with TRAIL at the cell surface.

Previously, we and others have reported on an approach that may be particularly useful in this respect. In brief, recombinant soluble TRAIL (sTRAIL) was genetically linked to a tumor cell–selective antibody fragment (scFv). The resultant recombinant scFv:TRAIL fusion protein has the following properties: By virtue of the antibody fragment, the scFv:TRAIL fusion protein selectively delivers TRAIL to the cell surface of tumor cells. Subsequently, cell surface bound scFv:TRAIL can activate TRAIL receptor apoptotic signaling in tumor cells (reviewed in ref. 13). Here, we utilized and adapted this approach for the selective delivery of high levels of TRAIL to the cell surface of...
T cells, with the aim of expanding the cytotoxic armament of these T cells with TRAIL (for schematic, see Fig. 1).

This approach was preclinically evaluated using 2 novel recombinant fusion proteins, designated anti-CD3:TRAIL and K12:TRAIL, that selectively bind to the T-cell surface antigen CD3 and CD7, respectively. Fusion protein anti-CD3:TRAIL contains a CD3 stimulatory antibody fragment, whereas K12:TRAIL contains a soluble form of the CD7 ligand K12. Anti-CD3:TRAIL and K12:TRAIL strongly potentiated the tumoricidal activity of T cells toward a panel of cancer cell lines, primary patient-derived malignant cells, and in a murine xenograft model. This strategy may represent an easy-to-incorporate approach to enhance the efficacy of various forms of adoptive T-cell therapy in cancer.

Materials and Methods

Reagents

Anti-CD7 mAb TH-69 competes with K12:TRAIL for binding to CD7 and was a gift from Prof. Dr. Martin Gramatzki (University of Kiel, Kiel, Germany). TRAIL-neutralizing mAb 2E5 was purchased from Kordia Life Translational Relevance

Because adoptive T-cell strategies generally fail to trigger clinically relevant anticancer immunity in patients with solid tumors, new strategies that optimize adoptive T-cell transfer are warranted. Here, we present a novel strategy that aims to selectively and safely enhance the antitumor activity of T cells by targeted delivery of human TNF-related apoptosis-inducing ligand (TRAIL) to the cell surface, whereby T cells can utilize TRAIL as an additional cytotoxic effector. This approach strongly enhances the antitumor activity of T cells toward a panel of cancer cell lines and primary patient-derived cancer cells. In addition, targeted delivery of TRAIL to the T-cell surface significantly extended the survival time of tumor-bearing mice. The approach reported here could also be easily integrated in current adoptive T-cell strategies.
Geneticin. HCT-116–luc was from Caliper Biosciences. DED cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). A375M.FADD-DED cells were generated by transfection of parental A375M cells with a dominant-negative FADD-DED construct using Fugene (Roche BV). A375M.FADD-DED cells were generated by transfection of parental A375M cells with a dominant-negative FADD-DED construct using Fugene (Roche BV). A375M.FADD-DED cells were generated by transfection of parental A375M cells with a dominant-negative FADD-DED construct using Fugene (Roche BV). A375M.FADD-DED cells were generated by transfection of parental A375M cells with a dominant-negative FADD-DED construct using Fugene (Roche BV). A375M.FADD-DED cells were generated by transfection of parental A375M cells with a dominant-negative FADD-DED construct using Fugene (Roche BV). A375M.FADD-DED cells were generated by transfection of parental A375M cells with a dominant-negative FADD-DED construct using Fugene (Roche BV).

Production of K12:TRAIL and anti-CD3:TRAIL

Fusion protein K12:TRAIL and anti-CD3:TRAIL were constructed by cloning the cDNA of soluble human K12 (aa 29–143) or the anti-CD3 antibody fragment scFvUCHT-1v9 in frame with human soluble TRAIL into previously described vector pEE14, yielding plasmids pEE14-K12:TRAIL and pEE14-anti-CD3:TRAIL, respectively. Fusion proteins K12:TRAIL and anti-CD3:TRAIL were produced in CHO-K1 cells essentially using previously described methods (14). Culture medium containing K12:TRAIL or anti-CD3:TRAIL was cleared by centrifugation (10,000 × g, 10 minutes), filter sterilized, and stored at −80°C until further use. Fusion proteins were purified via the N-terminal hemagglutinin (HA) tag using anti-HA affinity chromatography. Fusion protein anti-MCSF-TRAIL is a fusion protein that is essentially identical to anti-CD3:TRAIL except that it contains an anti-MCSF scFv instead of an anti-CD3 scFv. Melanoma-associated chondroitin sulfate proteoglycan (MCSP) is expressed on cells of the melanocyte lineage but not on T cells or carcinoma cells.

Cell lines

SK-OV-3, OvCAR-3, A2780, HCT-8, HT29, A375M, A2058, SK-MEL-28, HEP3B, PLC-PRF-5, FADU, PC-3M, HK-2, and Ramos were obtained from the American Tissue Culture Collection and characterized by short tandem repeat profiling, karyotyping, and isoenzyme analysis. A375M.FADD-DED cells were generated by transfection of parental A375M cells with a dominant-negative FADD-DED construct using Fugene (Roche BV). A375M.FADD-DED cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 500 µg/mL Geneticin. HCT-116–luc was from Caliper Biosciences (Xenogen). NHDF-juv cells were from Promocell. Human umbilical vein endothelial cells (HUVECs) were isolated as described previously. Unless otherwise indicated, cells were cultured in standard RPMI (Cambrex BioScience) supplemented with 10% FCS.

Isolation of primary patient-derived tumor cells, T cells and activation of T cells

Experiments were approved by the local Medical Ethical Committee and patients/healthy volunteers signed for informed consent. Tumor tissues and ascitic fluids were obtained during surgery procedures and if necessary minced, after which adherent cells were cultured. Experiments were carried out before passage number 5. Samples with a background apoptosis more than 30% were excluded from analysis. Normal fibroblast cultures originating from tumor tissue/ascitis were essentially established as described above for primary cancer cell cultures. Tumor-infiltrating T cells were isolated from the primary tumor cultures using ammonium chloride lysis. Peripheral blood lymphocytes (PBL) from blood of healthy donors were isolated using standard density gradient centrifugation (Lymphoprep; Axis-Shield PoC As). Activated T cells were generated by culturing PBLs with anti-CD3 mAb (0.5 µg/mL; 72 hours) and IL-2 (100 ng/mL; 48 hours).

Analysis of fusion protein binding and TRAILR expression

The presence of cell surface bound TRAIL was assessed by incubating cells with PE-conjugated anti-TRAIL mAb B-S23 (Diaclone SAS) for 60 minutes at 4°C followed by 3 washes. Cells were subsequently measured on an Accuri C6 flow cytometer. Cell surface display of TRAIL in time was determined by incubating cells with 50 pg per T cell K12:TRAIL or anti-CD3:TRAIL for 60 minutes at 4°C followed by 3 washes in PBS. Cells were then incubated at 37°C for the time points indicated and TRAIL expression was determined by flow cytometry using PE-labeled mAb B-S23. The amount of K12:TRAIL or anti-CD3:TRAIL bound to an individual T cell was determined using TRAIL ELISA. Briefly, 1 × 10^6 T cells were incubated with increasing concentrations of K12:TRAIL or anti-CD3:TRAIL for 1 hour. Subsequently, cells were centrifuged and supernatants used for TRAIL ELISA. Loss of K12:TRAIL and anti-CD3:TRAIL from the supernatant was calculated using the same concentration range of K12:TRAIL and anti-CD3:TRAIL in the absence of T cells as a reference. Expression of TRAIL receptors was determined by incubating cells with mAb HS-201, HS-202, HS-203, or HS-204 for 60 minutes at 4°C followed by 3 washes with PBS and incubation with PE-labeled goat anti-mouse Ab for 60 minutes at 4°C. Cells were subsequently washed 3 more times with PBS and TRAILR expression was determined using an Accuri C6 flow cytometer.

Analysis of cell death

For cell death assays, cancer cells were labeled with red fluorescent membrane label DiI (Invitrogen) in serum-free medium for 15 minutes followed by 3 washes in serum containing medium and subsequent culture in a 48-well
plate (3.0 × 10^4 cells per well). After 24 hours, cancer cells were treated for 16 hours with T cells (activated or resting) at the indicated ratios in the presence or absence of K12:TRAIL (50 pg per T cell), anti-CD3:TRAIL (50 pg per T cell), or anti-McSP:TRAIL (50 pg per T cell) after which apoptosis was assessed in tumor cells by loss of mitochondrial membrane potential (ΔΨm), as previously described (14) using an Accuri C6 flow cytometer. Where indicated, caspase inhibitors were used at a final concentration of 10 μmol/L. Actinomycin D was used at a final concentration of 2 μg/mL. Anti-TRAIL (2E5)- and anti-FlasL (Alf2.1)-neutralizing mAbs and TNF-α-neutralizing fusion protein Enbrel were used at a final concentration of 5 μg/mL. For experiments involving VPA, tumor cells were pretreated for 24 hours with 1 mmol/L of VPA prior to addition of T cells at the indicated ratios. For experiments involving concanamycin A (CMA), T cells were treated with the indicated concentrations of CMA for 2 hours prior to addition to tumor cells. Tumor cells were subsequently treated with these T cells for 4 hours in the presence of CMA after which induction of apoptosis was assessed. For outgrowth experiments using patient ascitic fluid, anti-CD3:TRAIL, anti-CD7 mAb, or rTRAIL were added at a final concentration of 100 ng/mL to a mixture of ascitic fluid and normal culture medium (50% v/v).

**Time lapse confocal microscopy**

Tumor cells were labeled with membrane label DiO (green) by incubation of 1 × 10^6 cells with 5 μL DiO in serum-free RPMI 1640 for 15 minutes at 37°C. Cells were then washed 3 times with RPMI 1640 + 10% FCS and DiO-labeled tumor cells were cultured in a glass coverslide (3.0 × 10^6 cells per well). After 24 hours, T cells labeled with Dil (red) using the same procedure were added to the tumor cells at the indicated effector-to-target (E:T) ratios. Time lapse confocal microscopy was carried out on a Solamere Nipkow Disk CLSM equipped with temperature/CO2-controlled cabinet and multicolor track table.

**Mechanical stimulation of CD3 signaling and calcium flux**

Glass coverslides in Petri dishes were coated overnight with 10 μg/mL TRAILR1-Fc, TRAILR2-Fc, TRAILR3-Fc, or TRAILR4-Fc. Coverslides were subsequently washed with PBS and blocked with normal human serum for 1 hour. T cells were labeled with FLUO-3-AM by incubating the cells with 5 μmol/L FLUO-3-AM ester in 100 μL PBS for 15 minutes at 20°C, followed by addition of 400 μL PBS with 10% FCS and 45 minutes of incubation at 37°C. Next, FLUO-3-AM labeled T cells were added to the TILL-TRAIL-R1-, TRAIL-R2-, TRAIL-R3-, or TRAIL-R4-coated glass slides. T cells were mechanically stimulated by using a micropipette, as previously described (15). Calcium flux was measured and quantified on a TILL iMIC fluorescence microscope equipped with temperature/CO2-controlled cabinet. Calcium flux is depicted as the mean fluorescent intensity (MFI) at any given time point (MFI_t)/the MFI at T = 0 (MFI_0).

**Cytokine ELISA**

T cells were incubated with tumor cells for 16 hours at an E/T ratio of 5:1 in the presence or absence of 50 pg per T cell anti-CD3:TRAIL. Where indicated, mAb 2E5 was added to block TRAIL–TRAILR interaction. Alternatively, T cells were treated with anti-CD3 mAb for 16 hours to elicit T-cell activation and cytokine release. Cell supernatants were subsequently harvested, centrifuged, and used for cytokine ELISA according to the manufacturer's instructions.

**HCT-116-luc xenograft mouse model**

Experiments were approved by the Committee for Research and Animal Ethics of the UMCG. Male athymic mice (Harlan) were intraperitoneally inoculated with HCT-116-luc (1 × 10^6 cells). Animals were subsequently treated with a single intraperitoneal dose of 5 × 10^7 T cells and about 150 μg/kg K12:TRAIL or anti-CD3/TRAIL, as indicated. Tumor growth was monitored by luminescent imaging of mice from the ventral abdominal side using an IVIS Spectrum (Xenogen) optical imager. Animals were sacrificed by cervical dislocation when bioluminescent signal was increased 10-fold compared with day of inoculation. For localization of T cells, T cells were labeled with CellVue NIR815 membrane labeling kit according to the manufacturer's instructions. Specific fluorescent signal was determined by subtracting the fluorescent signal from saline-treated mice.

**Statistical analysis**

Data reported are mean values ± SD of 3 independent experiments. Data was analyzed by 1-way ANOVA followed by Tukey–Kramer post test or, where appropriate, by 2-sided unpaired Student’s t test. Where indicated, *, P < 0.05; **, P < 0.01; ***, P < 0.001.

**Results**

**Tumoricidal activity of T cells is potently augmented by delivery of TRAIL to the T-cell surface**

To selectively deliver TRAIL to the cell surface of T cells, the CD3-targeted fusion protein anti-CD3:TRAIL and the CD7-targeted fusion protein K12:TRAIL were generated. In line with literature, ex vivo activated T cells obtained from healthy volunteers did not detectably express TRAIL (Fig. 2A). Incubation of these TRAIL-negative T cells with anti-CD3:TRAIL or K12:TRAIL lead to the display of high levels of TRAIL on the T-cell surface (Fig. 2A). A subsequent TRAIL ELISA showed that 1 × 10^5 T cells bind about 50 pg of K12:TRAIL and/or about 4 pg of anti-CD3:TRAIL. This T-cell binding by anti-CD3:TRAIL or K12:TRAIL was selectively blocked by anti-CD3 or anti-CD7 mAb, respectively (data not shown) and did not induce reciprocal T-cell death (Supplementary Fig. S1A).

Importantly, T-cell–selective binding by anti-CD3:TRAIL and K12:TRAIL did potentiate the tumoricidal activity of activated T cells toward cancer cells, with more than 90% apoptosis at an E/T ratio of 2:1 (Fig. 2B). Activated T cells alone failed to induce apoptosis at these E/T ratios,
Figure 2. Tumoricidal activity of T cells is potently augmented by delivery of TRAIL to the T-cell surface. A, TRAIL expression on anti-CD3+IL-2–activated T cells was assessed by incubation of T cells with PE-conjugated anti-TRAIL mAb (thin line). Cell surface TRAIL expression after binding of K12:TRAIL or anti-CD3:TRAIL was determined by preincubating T cells with K12:TRAIL (hashed line) or anti-CD3:TRAIL (solid line). Shaded gray area represents fluorescence of PE-labeled isotype control. B, activated T cells were incubated with OvCAR-3 cells in the presence or absence of K12:TRAIL (50 pg per T cell) or anti-CD3:TRAIL (50 pg per T cell) for 16 hours at the indicated E:T ratios and apoptosis was assessed in OvCAR-3 cells. C, activated T cells were incubated with OvCAR-3 cells in the presence or absence of K12:TRAIL (50 pg per T cell) or anti-CD3:TRAIL (50 pg per T cell) at an E:T ratio of 10:1 for the indicated time points and apoptosis was assessed in OvCAR-3 cells. D, cancer cell lines SK-OV-3, OvCAR-3, A2780, HCT-116, HCT-8, A549, A549, SK-MEL-28, HEP3B, PLC-PRF-5, FADU, PC-3M, and Ramos were treated for 16 hours with activated T cells at an E:T ratio of 5:1 in the presence or absence of K12:TRAIL (50 pg per T cell) or anti-CD3:TRAIL (50 pg per T cell) and apoptosis was assessed. E and F, primary ovarian cancer (OC) cells were treated as indicated at an E:T ratio of 5:1 for 16 hours and apoptosis was assessed. G, primary tumor cells were fluorescently labeled using membrane label DiO (green) and incubated with DiI (red)-labeled activated T cells in the presence or absence of K12:TRAIL (50 pg per T cell) at an E:T ratio of 10:1. Subsequently, cells were used for live fluorescent imaging (5 hours) to observe morphologic changes. For purpose of clarity, only cancer cells are depicted. See also Supplementary Videos S1–S4. n.s., nonsignificant.
expression and sensitivity to K12:TRAIL-mediated T-cell activity, TRAILR expression levels were determined in a panel of cell lines (Supplementary Fig. S1B).

This enhanced tumoricidal activity toward OvCAR-3 was dose dependent (Supplementary Fig. S1C and D), apparent within 2 hours and progressed to a maximum effect within 16 hours (Fig. 2C and Supplementary Videos S1 and 2). In addition, while the display of K12:TRAIL and anti-CD3:TRAIL on the T-cell surface was reduced over time (Supplementary Fig. S1E and F, respectively), T cells preincubated with excess K12:TRAIL or anti-CD3:TRAIL prior to addition to tumor cells (up to 48 hours) fully retained their enhanced apoptotic activity (Supplementary Fig. S1G). This indicates that in the presence of normal receptor turnover, sufficient K12:TRAIL or anti-CD3:TRAIL remain displayed on the T-cell surface. Importantly, the potentiating effect of anti-CD3:TRAIL and K12:TRAIL on the tumoricidal activity of T cells was detected in a panel of 14 solid tumor cell lines (Fig. 2D) and in an extensive panel of short-term cultures of primary patient-derived cancer cell samples (Fig. 2E and F; anti-CD3:TRAIL and K12:TRAIL, respectively). In these patient-derived malignant cell cultures, treatment with anti-CD3:TRAIL or K12:TRAIL and T cells, completely disrupted the monolayer within 5 hours as exemplified for K12:TRAIL in Figure 2G and Supplementary Videos S3 and 4.

To assess for possible effects of TRAIL receptor expression on the sensitivity of tumor cells to the potentiating effect of K12:TRAIL and anti-CD3:TRAIL on T-cell activity, TRAILR expression levels were determined in a panel of cell lines (see Table 1). There was no correlation between TRAILR expression and sensitivity to K12:TRAIL-mediated T-cell cytotoxicity (Supplementary Fig. S2A–D). Interestingly, there was a weak negative correlation ($r^2 = 0.48$) with TRAIL-R4 expression in terms of sensitivity to anti-CD3:TRAIL–mediated T-cell cytotoxicity (Supplementary Fig. S2D) but no correlation with TRAIL-R1, TRAIL-R2, and TRAIL-R3 (Supplementary Fig. S2A–C).

Taken together, anti-CD3:TRAIL and K12:TRAIL both potently augment the tumoricidal activity of activated T cells toward cancer cell lines and patient-derived malignant cells by about 500-fold.

### Table 1. MFIs for TRAILR expression levels determined on a panel of cancer cell lines

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**NOTE:** Values are representative from 3 independent experiments.

Anti-CD3:TRAIL induces tumoricidal activity by activation of resting T cells

Anti-CD3:TRAIL comprises a T-cell–stimulating CD3 antibody fragment. By virtue of this antibody fragment, anti-CD3:TRAIL might also be able to activate intrinsic cytotoxic effector mechanisms of T cells. In line with this hypothesis, resting T cells that lacked activity in coculture experiments with OvCAR-3 gained potent cytotoxic activity in the presence of anti-CD3:TRAIL (Fig. 3A, 100% apoptosis at E:T ratio of 10:1). In contrast, resting T cells remained ineffective in the presence of K12:TRAIL, with no cytotoxicity even at the highest E:T ratio tested (Fig. 3A). Furthermore, anti-CD3:TRAIL consistently reduced long-term outgrowth by more than 50% in ascitic fluid from ovarian cancer patients that contains both primary malignant cells and autologous T cells (Fig. 3B and Supplementary Fig. S3A). Neither an anti-CD3 stimulatory mAb nor an untargeted soluble rhTRAIL preparation alone inhibited cancer cell outgrowth (Fig. 3B).

In line with these activating properties, anti-CD3:TRAIL induced a rapid calcium flux in T cells cocultured with OvCAR-3 cells (Supplementary Fig. S3B) which was followed in time by an increase in the number of T cells positive for the activation marker CD69 (Supplementary Fig. S3C) and the release of proinflammatory T-cell cytokines (Supplementary Fig. S3D). Interestingly, preventing cellular contact between T cells and tumor cells abolished T-cell activation induced by anti-CD3:TRAIL (Supplementary Fig. S3E), as did the addition of TRAIL-neutralizing and TRAILR-blocking antibodies (Supplementary Fig. S3C). In contrast, caspase inhibition did not block activation of anti-CD3:TRAIL–armed T cells (Supplementary Fig. S3C) but did block induction of apoptosis by rhTRAIL (Supplementary Fig. S5A). Further analysis using T cells mechanically manipulated using a micropipette (Supplementary Fig. S4A for schematic representation) indicated that anti-CD3:TRAIL–mediated T-cell activation was likely because of shear stress upon simultaneous binding of anti-CD3:TRAIL to CD3 on T cells and TRAILR2 on tumor cells (Supplementary Fig. S4A–C).

K12:TRAIL and anti-CD3:TRAIL enhance tumoricidal activity of resting T cells via distinct mechanisms

On the basis of these activating properties, T-cell cytotoxicity induced by anti-CD3:TRAIL may, at least partly, be attributable to stimulation of intrinsic T-cell cytotoxicity. Indeed, the potentiating effect on T-cell activity by...
Figure 3. K12:TRAIL and anti-CD3:TRAIL enhance tumoricidal activity of resting T cells via distinct mechanisms. A, OvCAR-3 cells were treated with resting T cells at the indicated E:T ratios for 48 hours in the presence or absence of K12:TRAIL (50 pg per T cell) or anti-CD3:TRAIL (50 pg per T cell) and apoptosis was assessed. B, ascitic fluid (n = 5) was mixed with culture medium at a 1:1 ratio and treated with anti-CD3:TRAIL (50 pg per T cell), anti-CD3 mAb (2 μg/mL), or rhTRAIL (100 ng/mL) for 12 days after which cell viability was assessed. C, OvCAR-3 cells were incubated for 4 hours at an E:T of 10:1 with activated T cells ± K12:TRAIL (50 pg per T cell) or anti-CD3:TRAIL (50 pg per T cell) in the presence of the indicated concentrations of CMA and apoptosis was assessed. D, A375M or A375M.FADD-DED cells were treated with TRAIL (100 ng/mL), act T cells (5:1) ± K12:TRAIL (50 pg per T cell), or anti-CD3:TRAIL (50 pg per T cell) and apoptosis was assessed. E, OvCAR-3 cells were treated for 16 hours with activated T cells ± K12:TRAIL (50 pg per T cell) or anti-CD3:TRAIL (50 pg per T cell) at an E:T ratio of 10:1 in the presence or absence of TRAIL-neutralizing mAb 2E5, FasL-neutralizing mAb Alf2.1, TNF-α-neutralizing fusion protein Enbrel, pan-caspase inhibitor zVAD-fmk, caspase-8 inhibitor zIETD-fmk, or caspase-9 inhibitor zLEHD-fmk and apoptosis was assessed.

Figure 4. K12:TRAIL and anti-CD3:TRAIL inhibit growth of colorectal carcinoma in vivo. A, male athymic mice were injected intraperitoneally with 1 x 10^6 HCT-116-luc cells followed by intraperitoneal injection with saline, activated T cells (5 x 10^5), K12:TRAIL (0.25 mg/kg), anti-CD3:TRAIL (0.25 mg/kg), activated T cells ± K12:TRAIL, or activated T cells ± anti-CD3:TRAIL and tumor growth was monitored by bioluminescence. Mice were sacrificed when tumor size reached a 10-fold increase compared with initial tumor load. Representative images of mice from all treatment groups on days 3 and 14 after tumor cell injection are depicted. B, mice survival following treatment with act. T cells, K12:TRAIL, or act. T cells + K12:TRAIL, as determined by a 10-fold increase in tumor size compared with start of treatment, was plotted in a Kaplan–Meier curve. C, mice survival following treatment with act. T cells, anti-CD3:TRAIL, or act. T cells + anti-CD3:TRAIL, as determined by a 10-fold increase in tumor size compared with start of treatment, was plotted in a Kaplan–Meier curve. D, male athymic mice were injected intraperitoneally with 1 x 10^6 HCT-116-luc cells followed by intraperitoneal injection with saline, activated T cells (1 x 10^5), activated T cells + K12:TRAIL (0.25 mg/kg), or activated T cells + anti-CD3:TRAIL (0.25 mg/kg). T-cell localization was subsequently monitored at the indicated time points using fluorescence together with tumor localization using bioluminescence. See also Supplementary Videos 5 and 6.
Enhancing T-cell Tumoricidal Activity with TRAIL

A

B

C

D

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www.aacrjournals.org Clin Cancer Res; 17(17) September 1, 2011

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Published OnlineFirst July 13, 2011; DOI: 10.1158/1078-0432.CCR-11-0303

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anti-CD3:TRAIL, but not by K12:TRAIL, was dose dependently blocked by the granzyme/perforin release inhibitor CMA (Fig. 3C and Supplementary Fig. S5B). In addition, A375M cells overexpressing a mutant form of the TRAILR adaptor protein FADD (FADD-DED) that inhibits TRAIL-mediated caspase activation, remained sensitive to treatment with T cells + anti-CD3:TRAIL but not to T cells + K12:TRAIL (Fig. 3D). Inhibition of other typical cytotoxic T-cell effectors, such as the tumoricidal proteins Fasl and TNF-α, failed to inhibit apoptotic activity of anti-CD3:TRAIL/K12:TRAIL toward OvCAR-3 cells (Fig. 3E). Apoptotic activity of anti-CD3:TRAIL/K12:TRAIL–armed T cells was abrogated by a pan-caspase inhibitor (Fig. 3E) and by inhibition of caspase-8 or caspase-9 (Fig. 3E). Of note, the potentiating effect of anti-CD3:TRAIL on tumoricidal T-cell activity closely resembled that of T cells redirected with the bispecific antibody Bis-1 (anti-CD3:anti-EpCAM), which was also abrogated by CMA (Supplementary Fig. SSC). Therefore, the mode of action of anti-CD3:TRAIL appears to be largely dependent on granzyme/perforin-mediated lysis, whereas K12:TRAIL functions via activation of initiator caspase-8/-9 and effector caspases. Furthermore, the inhibitory effect of caspase-9 blockade suggests that mitochondrial amplification of the caspase-8 apoptotic signal is required for effective tumoricidal activity.

**K12:TRAIL and anti-CD3:TRAIL inhibit growth of colorectal carcinoma in vivo**

The potent in vitro effects of K12:TRAIL and anti-CD3:TRAIL prompted us to evaluate activity against tumor cells in vivo. To this end, we xenografted colorectal carcinoma cell line HCT-116–luc intraperitoneally in mice as a model for advanced metastatic colon carcinoma. HCT-116–luc cells rapidly established tumor nodules throughout the abdominal cavity, resulting in a median survival of only 7 days (Fig. 4B and C). Importantly, growth of HCT-116–luc in vivo was significantly suppressed by cotreatment with K12:TRAIL and activated T cells, compared with treatment with either K12:TRAIL or T cells alone. Indeed, median survival was increased 6-fold and 1 mouse survived for the duration of the experiment (70 days) with minimal residual disease (Fig. 4A and B; 42 vs. 7 days). Of note, cotreatment with K12:TRAIL and T cells did not have obvious off-target toxicity in these mice, with no apparent weight loss (data not shown). In addition, fibroblasts (NHDF-juv), normal kidney cells (HK-2), and endothelial cells (HUVEC) were resistant to cotreatment with K12:TRAIL and T cells (Supplementary Fig. S5D). Similarly, cotreatment of mice with T cells and anti-CD3:TRAIL resulted in a striking increase in median survival with 4 of 5 mice remaining tumor free for the duration of the experiment (Fig. 4A and C). Treatment with anti-CD3:TRAIL alone had no effect on animal survival (Fig. 4C). Of note, while no deleterious effects on animal well being were observed during the course of the experiment, the in vitro treatment of NHDF-juv, HK-2, and HUVECs with T cells and anti-CD3:TRAIL did trigger an increase in the percentage of apoptosis (Supplementary Fig. S5D).

**K12:TRAIL and anti-CD3:TRAIL are compatible with experimental strategies to ameliorate GVHD and optimize GVT**

Earlier studies have shown that TRAIL expression on T cells is required for optimal GVT activity during AHCT but does not exacerbate GVHD. Therefore, it was examined whether K12:TRAIL could be used in conjunction with therapies currently being evaluated for optimizing AHCT. In particular, the so-called histone deacetylase inhibitors (HDACi) inhibit GVHD in preclinical settings (16, 17) but are also well known to synergize with the proapoptotic activity of TRAIL (18, 19). Of note, HDACi do not potentiate the GVT effect. In line with this, pretreatment with HDACi VPA did not enhance the tumoricidal activity of T cells alone (Fig. 5A). In contrast, pretreatment of OvCAR-3 cells with VPA strongly optimized the potentiating effect of K12:TRAIL and anti-CD3:TRAIL on tumoricidal T-cell activity, with approximately 30% and 50% cell death in OvCAR-3 at E:T ratio of 1:50, respectively (Fig. 5A–C). Interestingly, VPA enhanced the antitumor effect of K12:TRAIL, but not anti-CD3:TRAIL, toward 2 patient-derived tumor samples (Fig. 5D).

Various other strategies have been developed to selectively enhance GVMT or ameliorate GVHD without affecting GVT. These strategies include the selective depletion of a population of alloreactive CD69high T cells before infusion (20) or selectively transferring only CD4+CD25+, CD8+, or CD25+ cells (21). K12:TRAIL and anti-CD3:TRAIL potentiated the tumoricidal activity of isolated CD69low, CD69medium, or CD69high T-cell populations to a similar degree (Fig. 5F). Similarly, K12:TRAIL and anti-CD3:TRAIL potentiated the tumoricidal activity of sorted CD4+ and CD8+ T cells to a level similar to that observed in nonsorted T cells (Fig. 5G). Therefore, K12:TRAIL and anti-CD3:TRAIL appear suitable for integration in existing strategies for optimizing AHCT.

**Discussion**

Here, we report on the preclinical evaluation of a new strategy designed to deliver high levels of the proapoptotic effector molecule TRAIL to the surface of T cells and thereby safely augment antitumor T-cell activity. Arming T cells with anti-CD3:TRAIL or K12:TRAIL resulted in a more than 500-fold increase in tumoricidal activity toward both colorectal xenografts. To determine whether T cells localized to the intraperitoneally growing tumor cells, fluorescently labeled T cells were followed up to 5 days in an intraperitoneal tumor model with HCT-116–luc (Fig. 4D). Treatment with activated T cells or activated T cells + anti-CD3:TRAIL resulted in colocalization of bioluminescent (tumor) and fluorescent (T cell) signal in tumor nodules in the abdominal cavity after 2 days but not after 5 days (Fig. 4D). Upon treatment with activated T cells + K12:TRAIL (Fig. 4D), the T cells were found to colocalize with tumor signal until tumor cells were eliminated (Fig. 4D). Taken together, K12:TRAIL and anti-CD3:TRAIL potently enhance antitumor activity of T cells and inhibit growth of colorectal xenografts in vivo.
Figure 5. K12:TRAIL and anti-CD3:TRAIL are compatible with experimental strategies to ameliorate GVHD and optimize GVT. A, OvCAR-3 cells were pretreated with VPA (1 nmol/L) for 1 day and subsequently treated for 16 hours with activated T cells at the indicated E:T ratios. B, OvCAR-3 cells pretreated with VPA for 1 day were treated for 16 hours with K12:TRAIL–armed T cells (50 pg per T cell) at the indicated E:T ratios. C, OvCAR-3 cells pretreated with VPA for 1 day were treated for 16 hours with anti-CD3:TRAIL–armed T cells (50 pg per T cell) at the indicated E:T ratios. D, primary cancer cells (n = 2) were pretreated with VPA (1 nmol/L) for 1 day and subsequently treated for another 16 hours with activated T cells or K12:TRAIL/anti-CD3:TRAIL–armed T cells (50 pg per T cell) at an E:T ratio of 5:1. E, activated T cells were double stained for CD3 and CD69, whereupon CD69low, CD69medium, and CD69high populations were sorted from the CD3-positive cells. F, OvCAR-3 cells were treated with CD69low, CD69medium, and CD69high and corresponding K12:TRAIL/anti-CD3:TRAIL–armed T-cell populations (50 pg per T cell) for 16 hours at an E:T ratio of 1:1. G, activated T cells were separated into CD4 and CD8 populations, whereupon OvCAR-3 cells were treated with the different T-cell populations and K12:TRAIL/anti-CD3:TRAIL–loaded populations (50 pg per T cell) for 16 hours at an E:T ratio of 5:1.
are also known to sensitize cancer cells to TRAIL-induced inhibitors, such as HDACi (16, 17) or rapamycin (23), to selectively enhance GVT. Interestingly, novel GVHD the data of the current study suggest that targeted delivery contribute to GVHD (10). Although not investigated here, are the primary mediators of GVT activity as well as GVHD. would be in the context of AHCT. Alloreactive donor T cells tion of T-cell–specific delivery of TRAIL using K12:TRAIL during AHCT. Indeed, perhaps the most obvious applica-
neered T cells, T-cell bodies, as well as in GVT activity specific kind of T-cell immunotherapy but may be applied to ex vivo expanded tumor infiltrating lymphocytes, engi-
erroded T cells, T-cell bodies, as well as in GVT activity during AHCT. Indeed, perhaps the most obvious applica-
tion of T-cell–specific delivery of TRAIL using K12:TRAIL would be in the context of AHCT. Alloreactive donor T cells are the primary mediators of GVT activity as well as GVHD. Earlier studies have shown that TRAIL expression on T cells was required for optimal GVT, whereas TRAIL did not contribute to GVHD (10). Although not investigated here, the data of the current study suggest that targeted delivery of TRAIL to donor T cells using K12:TRAIL may be of value to selectively enhance GVT. Interestingly, novel GVHD inhibitors, such as HDACi (16, 17) or rapamycin (23), are also known to sensitize cancer cells to TRAIL-induced apoptosis (18, 19). In line with this, our experiments show further optimization of the potentiating effect of K12: TRAIL by pretreatment of tumor cells with the HDACi VPA. Thus, rationally designed combinatorial strategies that include K12:TRAIL may well yield optimal GVT activity with no/minimal GVHD.

The here described immunotherapeutic approach to optimize T-cell immunotherapy may be further explored using TRAIL fusion proteins that incorporate other immunomodulatory scFvs or ligands that would simultaneously provide stimulatory signals to T cells and add TRAIL signal-
ing to the T-cell armamentarium. Likely combinations to this effect include stimulatory anti-CD28 scFvs (24) or inhibitory scFvs directed against cytotoxic T-lymphocyte antigen 4 (CTLA-4; ref. 25). In this respect, targeted activa-
tion of CTLA-4 signaling using a fusion protein CTLA-4: TRAIL has been shown to limit T-cell activity and amelio-
rate autoimmunity in preclinical models (26). Reversely, inhibition of CTLA-4 signaling may potentiate antitumor T-cell activity.

In conclusion, cell surface delivery of TRAIL to T cells potently augments the antitumor activity of T cells in vitro and in vivo and might be widely applicable in current/experimental T-cell–based strategies to optimize anticancer efficacy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Joan Vos and Niels Kouprie for their technical assistance.

Grant Support

This work was supported by the Dutch Cancer Society (grant numbers NUG 2009-3555 to E. Bremer and NUG2007-3784 to W. Helfrich), the Nether-
lands Organization for Scientific Research (E. Bremer), the Melanoma Research Alliance (E. Bremer), and the Alexander von Humboldt Foundation (E. Bremer).

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Received February 11, 2011; revised June 10, 2011; accepted July 1, 2011; published OnlineFirst July 13, 2011.


Cell Surface Delivery of TRAIL Strongly Augments the Tumoricidal Activity of T Cells

Marco de Bruyn, Yunwei Wei, Valerie R. Wiersma, et al.