IL13Rα2- and EGFR-targeted pseudomonas exotoxin potentiates the TRAIL-mediated death of GBM cells

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Abstract. Glioblastomas (GBMs) are refractory to current treatments and novel therapeutic approaches need to be explored. Pro-apoptotic tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is tumor-specific and has been shown to induce apoptosis and subsequently kill GBM cells. However, approximately 50% of GBM cells are resistant to TRAIL and a combination of TRAIL with other therapeutics is necessary to induce mechanism-based cell death in TRAIL-resistant GBMs. The present study examined the ability of the tumor cell surface receptor, interleukin (IL)-13 receptor α2 (IL13Rα2)- and epidermal growth factor receptor (EGFR)-targeted pseudomonas exotoxin (PE) to sensitize TRAIL-resistant GBM cells and assessed the dual effects of interleukin 13-PE (IL13-PE) or EGFR nanobody-PE (ENb-PE) and TRAIL for the treatment of a broad range of brain tumors with a distinct TRAIL therapeutic response. Receptor targeted toxins upregulated TRAIL death receptors (DR4 and DR5) and suppressed the expression of anti-apoptotic FLICE-inhibitory protein (FLIP) and X-linked inhibitor of apoptosis protein (XIAP). This also led to the induction of the cleavage of caspase-8 and caspase-9 and resulted in the sensitization of highly resistant established GBM and patient-derived GBM stem cell (GSC) lines to TRAIL-mediated apoptosis. These findings provide a mechanism-based strategy that may provide options for the cell-mediated delivery of bi-functional therapeutics to target a wide spectrum of TRAIL-resistant GBMs.

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Introduction

Glioblastoma (GBM) is the most common and aggressive type of brain tumor affecting humans, with the survival of patients being <2 years from the time of diagnosis (1-3). The hallmarks of GBM include molecular and cellular heterogeneity, uncontrolled proliferation, diffuse infiltration, necrosis, angiogenesis, resistance to apoptosis and genomic instability (4-7). Current treatment modalities involve a combination of maximal tumor resection, radiotherapy and concomitant chemotherapy; however, the median survival rate is 14.6 months (8). Toxicity in healthy tissue is a common side-effect and tumor recurrence is almost inevitable (9). There is therefore an urgent need for the development of novel therapies that act in a robust and GBM-selective manner.

One therapeutic candidate is tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a pro-apoptotic cytokine. TRAIL holds much potential as an anticancer therapeutic since it binds to death receptors (TRAIL-R1; DR4 and TRAIL-R2; DR5) that are exclusively expressed on cancerous cells. The binding of TRAIL induces the recruitment of Fas-associated death domain (FADD) adaptor protein, which in turn recruits procaspase-8 to the intracellular death domain of DR4/5 (10). Once cleaved, caspase-8 activates subsequent executioner caspases-3/7, leading to the activation of the extrinsic apoptotic cascade (11-13). Active caspase-8 can additionally induce the intrinsic apoptotic pathway via the cleavage of BH3 interacting-domain death agonist (BID). The truncated form, tBID, interacts with Bax and ultimately causes mitochondrial outer membrane permeabilization and activation of the caspase cascade via caspase-9 (14).

Although a number of established and patient-derived GBM cell lines are sensitive to TRAIL, approximately half of these display some degree of TRAIL resistance, thus limiting its therapeutic potential (12,15-17). To increase the effectiveness of TRAIL therapy, it is necessary to understand the mechanisms that cause TRAIL resistance and develop therapeutic approaches that can overcome resistance by sensitizing an otherwise TRAIL-resistant population of cells. This has been tested using a combination approach with either TRAIL-sensitizing agents, or biologics that target different

tumor-killing mechanisms to enhance death-inducing signals (16,18-20). One such approach was used by Horita *et al,* who treated GBMs with TRAIL in combination with an epidermal growth factor receptor (EGFR)-targeted diphtheria toxin (DT-EGF) (21). When TRAIL and DT-EGF were used in combination, a synergy in GBM cell death was observed compared to the cells treated with the monotherapies alone. This effect was caused by DT-EGF depleting FLICE-inhibitory protein (FLIP), an inhibitor of TRAIL-mediated apoptosis, thereby potentiating TRAIL-induced apoptosis (21).

The authors have previously engineered toxin-resistant somatic and human neural stem cells to continually secrete 2 pseudomonas exotoxin (PE)-cytotoxins, IL13-PE and ENb-PE, that target interleukin (IL)-13 receptor α2 (IL13Rα2) or EGFR, respectively (22), expressed by a number of GBMs (22-25). PE has a similar mechanism of action as DT, resulting in the inhibition of protein synthesis and the death of targeted cells (26-28). Previously, the authors demonstrated efficacy in multiple GBM lines, and established an association between GBM cell death and the expression of cognate receptors (22). It would be ideal to create a bifunctional stem cell (SC) that secretes PE-cytotoxin and TRAIL; this combination may prove effective by targeting multiple GBM subtypes and potentially sensitizing TRAIL-resistant populations. Furthermore, SC delivery would circumvent the short half-life, systemic toxicity and poor tissue penetration attributed to standard chemotherapeutic administration (9,29,30).

In the present study, it was demonstrated that the exposure of TRAIL-resistant GBM lines to targeted PE-cytotoxins led to the upregulation of DR4/5, and the depletion of the anti-apoptotic proteins, FLIP and XIAP, thereby breaking TRAIL resistance and sensitizing the cells to TRAIL. Furthermore, combining pro-apoptotic TRAIL with the protein synthesis inhibitory effects of PE-cytotoxins resulted in an enhanced GBM cell death by potentiating both the extrinsic and intrinsic apoptotic pathways.

Materials and methods

Preparation of lentiviral constructs. Based on the lentiviral transfer vectors, pLV-CICS/IG and pLV-CICS, the following therapeutic and diagnostic lentiviral vectors were engineered as previously described (13,31): i) LV-TRAIL' ii) LV-ENb-PE' iii) LV-IL13-PE; iv) LV-GFP/RLuc; v) LV-IL13Rα2-GFP/Rluc; vi) LV-Fluc/mCherry; and vii) LV-GFP/Fluc. Recombinant IL13-PE was constructed in the previously described Pico2 vector by replacing Fluc with IL13-PE (32). IL13 was PCR-amplified using pORF5-hIL13 (Invitrogen; Thermo Fisher Scientific, Inc.) as a template with primers encoding *Nhe1* and *PspX1*. The PCR fragment was ligated into *Nhe1*/*PspX1*-digested Pico2. To create IL13-PE, IL13 was PCR-amplified as described above with primers encoding *Nhe1* and *EcoRV*. PE, which lacks the receptor binding domain sequence (domain Ia and Ib), was amplified by PCR with primers encoding *EcoRV* and *PspX1* using pJH8 (ATCC) as a template. The two fragments were then ligated into *Nhe1*/*PspX1*-digested Pico2. To construct the cytotoxic variant of EGFR nanobodies, the cDNA encoding an 18-aa linker sequence (*lin*) and PE sequence were amplified by PCR and fused with the LV-ENb construct at the *EcoRV* and *Xho1* sites. The *EcorV-Linker*-PE-*Xho1* cDNA fragment were amplified with *EcoRVlin*PE forward and *Xho1*-PE reverse primers and directionally inserted in *EcoRV-Xho1*-digested LV-ENb, resulting in LV-ENb-PE. To obtain a clinically-proven lentiviral vector bearing IL13-PE-TRAIL, IL13-PE was first PCR-amplified from the LV-(CMV)-IL13-PE vector and then directionally sub-cloned into the previously described (31) LV-(EF)-Nb-TRAIL by replacing Nb fragment with IL13-PE (since the C terminal of TRAIL contains cytotoxic domain, we cloned IL13-PE in front of the N terminal of TRAIL sequence). Lentiviral constructs were packaged as lentiviral vectors in 293T/17 (ATCC) and in toxin-resistant 293 oligo cells for cytotoxin constructs by using a helper virus-free packaging system as previously described (31) and GBM cells were engineered to express florescence and bioluminescence imaging agents as previously described (32,33).

Cells and cell culture. The established GBM cell lines, LN229, U138 and U251, were purchased from ATCC and grown in glioma growth medium; DMEM supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin/streptomycin. The highly invasive tumor-initiating primary cell lines were grown in neural basal medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 3 mM L-glutamine, 2 μg/ml heparin, 20 ng/ml epidermal growth factor (EGF) and 20 ng/ml basic fibroblast growth factor (bFGF).

Establishment of bio-imageable tumor cells. GBM cells were transduced with either LV-Fluc/mCherry or LV-GFP/Fluc at a multiplicity of infection (MOI) of 2 and populations of transduced cells were visualized by fluorescence microscopy for imageable reporter gene expression (mCherry and GFP). To establish a pure cell line, transduced cells with lentiviral vectors were sorted for high expressers of GFP and mCherry using a FACSAria Ilu cell sorter (BD Biosciences). GBM cells were transduced with pico2 vector were selected with puromycin and sorted for high expressers of florescence proteins using a FACSAria Ilu cell sorter (BD Biosciences).

Dot blot quantification. Serum-free conditioned medium from 293 oligo cells engineered to express secretable forms of IL13-PE, ENb-PE and TRAIL were collected 24 h following transfection and concentrated with centrifugal filter units (Amicon, MilliporeSigma). GFP-conditioned medium was collected and used for each control treatment. Purified IL13 (Chemicon; Thermo Fisher Scientific, Inc.; 100 ng/μl) was used as a positive control for the quantification of IL13-PE. To determine the ENb-PE concentrations, IL13PE was used as a positive control. Concentrated condition medium (20X) was loaded on a nitrocellulose membrane (1 and 3 μl) and immunoblotted using antibodies against IL13 (cat. no. ab106732, Abcam; antibody dilution; 1:1,000) and PE (cat. no. P2318, Sigma-Aldrich; Merck KGaA; antibody dilution; 1:20,000) for IL13-PE and ENb-PE, respectively. The antibody incubations were performed at room temperature for 1 h on a shaker. Following primary antibody incubation, the membrane was also probed with HRP conjugated secondary antibodies (1:5,000) goat anti-mouse (cat. no. ab6789, Abcam) and goat anti-rabbit (cat. no. ab6702, Abcam) accordingly for 30 min at room temperature. Band intensities and relative concentration were quantified using NIH ImageJ software.

Cell viability assays. To examine the combined therapeutic effects of PE-fused cytotoxins and S-TRAIL *in vitro*, a panel of GBM lines was transduced with either LV-Rluc or LV-Fluc and seeded in 96-well plates (Matrical Bioscience). All patient derived GSC lines (BT74, GBM4, GBM8, GBM18, GBM23 and GBM64) used in the present study were previously established (34) and provided by Dr Wakimoto (Department of Neurosurgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA). A panel of cell lines were firstly applied to a viability assay for TRAIL and PE cytotoxin treatments. Both established TRAIL-resistant GBM lines and patient-derived GSC lines (GBM23 and GBM64) expressing the target receptors for PE cytotoxins were analyzed for TRAIL and PE cooperation. The cells were pre-treated with conditioned media containing 25 ng/ml of PE-cytotoxins and 500 ng/ml S-TRAIL (100 ng/ml S-TRAIL was added for TRAIL semi-sensitive lines) was subsequently added after 24 h of toxin exposure. Cell viability was measured with 15 μg/ml of D-luciferin (Biotium, Inc.) for the Fluc signal or 1 μg/ml coelanterazine (Nanolight) for the Rluc signal. For non-transduced cell lines, metabolic activity was measured using ATP-dependent luminescent reagent (CellTiter-Glo, Promega Corporation). For all *in vitro* assays, photon emission was measured using a cryogenically cooled high efficiency CCD camera system (Roper Scientific, Inc.).

BLI assay for apoptosis. To investigate the concentration dependency of PE-fused cytotoxins in combination with S-TRAIL on GBM viability and caspase activities, non-transduced GBM cells were treated as described above and measured using CellTiterGlo and CaspaseGlo 3/7, respectively (Promega Corporation) following manufacturer's guidelines (Promega Corporation). All experiments were performed in triplicate.

Western blot analysis

Receptor expression. Cell lysates from a panel of GBM cell lines were prepared using Nonidet P-40 lysis buffer containing complete protease inhibitor mixture (Roche Diagnostics) and protein amount was quantified using a BCA Protein Assay (Bio-Rad Laboratories, Inc.). The rotein amount in each sample was determined prior to the SDS treatments using a DC Protein assay kit (Bio-Rad Laboratories, Inc.) and a total of 25 μg protein samples were loaded in each well of 4-15% polyacrylamide (gradient) gels (Bio-Rad Laboratories, Inc.) and proteins were transferred onto nitrocellulose membranes by wet blotting in ice for 90 min. (Bio-Rad transfer system) The membranes were then blocked in 5% bovine serum albumin (BSA) at room temperature for 90 min. Proteins were examined by western blot analysis using either antibodies against EGFR (cat. no. D38B1, Cell Signaling Technology, Inc.; antibody dilution; 1:1,000) or IL13Rα2 (cat. no. AF146, R&D Systems, Inc.; antibody dilution, 0.2 μg/ml). The membranes were blocked in 5% BSA for 90 min. at room temperature and incubated with proper primary antibodies overnight at 4˚C.

Expression and secretion of therapeutics. The cell lysates and serum-free conditioned medium from 293.oligo-IL13-PE, or 293.oligo-ENb-PE or 293.oligo-GFP were collected following transduction (16 h following transduction, the infection medium was replaced with a serum-free medium and following 24 h of incubation at 37˚C 5% CO_2 incubator, the conditioned medium was collected) and examined by western blot analysis using antibodies against IL-13 (cat. no. ab106732, Abcam; antibody dilution, 1:1,000) or PE (cat. no. P2318, Sigma-Aldrich; Merck KGaA; antibody dilution, 1:20,000). The membranes were blocked in 5% BSA for 90 min and the antibody incubations were performed overnight at 4˚C. Following primary antibody incubation, all membranes were also probed with HRP-conjugated secondary antibodies (1:5,000) goat anti-mouse (ab6789, Abcam) and goat anti-rabbit (ab6702, Abcam) accordingly for 1 h at room temperature.

TRAIL sensitization upon targeted toxin mono-treatments. LN229 and LN229-IL13Rα2 cells were treated with ENb-PE and IL13-PE, respectively and cell lysates were analyzed with antibodies against β-actin (cat. no. 4967, Cell Signaling Technology, Inc.; antibody dilution, 1:2,000), DR4 (cat. no. 1139, ProSci; antibody dilution, 1 μg/ml), DR5 (cat. no. 2019, ProSci; antibody dilution, 1 μg/ml) and XIAP (cat. no. 14334, Cell Signaling Technology, Inc; antibody dilution, 1:20,00). All membranes were blocked in 5% BSA for 90 min at room temperature prior to antibody incubation. Following primary antibody incubation, all membranes were also probed with HRP-conjugated secondary antibodies (1:5,000) goat anti-mouse (ab6789, Abcam), goat anti-rabbit (ab6702, Abcam) and donkey anti-goat (ab182021, Abcam) accordingly for 1 h at room temperature.

Apoptosis on targeted toxin and TRAIL combined treatments. Patient-derived GSC (glioma stem cell) lines (GBM23 and GBM64) and established GBM lines (U251, LN229 and LN229-IL13Rα2) were pre-treated with PE-fused cytotoxins (25 ng/ml) for 24 h, then treated with 500 ng/ml S-TRAIL (100 ng/ml for U251 cells). Following 24 h of S-TRAIL exposure, cell lysates were analyzed using antibodies against β-actin (cat. no. 4967, Cell Signaling Technology, Inc.) and apoptotic markers including cleaved poly(ADP-ribose) polymerase (PARP) (cat. no. 5625, Cell Signaling Technology, Inc.), caspase-8 (cat. no. 9746, Cell Signaling Technology, Inc.) and caspase-9 (cat. no. 9508, Cell Signaling Technology, Inc.). All membranes were blocked in 5% BSA for 90 min at room temperature prior to antibody incubation. In this experimental setting, primary antibody incubations were performed at a 1:2,000 dilution for actin antibody and a 1:1,000 dilution for all other antibodies. Following primary antibody incubation, all membranes were also probed with HRP conjugated secondary antibodies (1:5,000) goat anti-mouse (ab6789, Abcam) and goat anti-rabbit (ab6702, Abcam) accordingly for 1 h at room temperature.

Data quantification. Protein bands on X-ray films were visualized with a chemiluminescence substrate (Super Signal™ West Pico PLUS; Thermo Fisher Scientific, Inc.) and the intensity of bands was quantified using NIH ImageJ software.

Reverse transcription PCR. RNA samples were extracted by using RNeasy kit (Qiagen, Inc.). Total RNA (0.5 μg) was reverse transcribed to obtain cDNA using the Superscript VILO cDNA synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.). A cDNA library was obtained after 30 cycles

of amplification (95˚C, 30 sec.-60˚C, 45 sec.-72˚C, 45 sec-72˚C, 10 min.) (Qiagen, Inc.) and Human IL13Rα2 chain was amplified using the primer pair (sense, 5'-ATGGCTTTCGTTTGC TTGGCTAT-3' and antisense, 5'-TCATGTATCACAGAA AAATTCTGG-3') yielding a product of 1,130 bp. A portion of PE was amplified using the primer pair (sense, 5'-GAACCC GACGCACGCGGCCGG-3' and antisense, 5'-CCGCTCGAG CTTCAGGTCCTCGCGCGGCG-3') to generate a 445 bp product. A human GAPDH primer pair (sense, 5'-GTCAGT GGTGGACCTGACCT-3' and antisense, 5'-TGCTGTAGC CAAATTCGTTG-3') was used yielding 245 bp PCR product as a positive control.

Immunofluorescence staining. GBM cells were plated onto a coverslip containing wells of 24 well plates $1x10^4$ cells/well and treated for 24 h with 25 ng/ml IL13-PE; or ENb-PE; or GFP (control) accordingly. The cells were then fixed with 4% PFA (paraformaldehyde) for 10 min at room temperature and blocked with 1X phosphate-buffered saline (PBS) including 5% NGS (normal goat serum) and 0.3% Triton-X at room temperature for 2 h. Primary antibody against Ki-67 (M7240, Dako; Agilent Technologies, Inc.) was added (1:100) to each well and incubated overnight at 4˚C. The following day, after washing with PBS, the cells were incubated with Alexa-647 conjugated goat anti-mouse antibody (ab150115, Abcam) and after washing with PBS, the cover slips were mounted with Vectashield mounting medium with DAPI (H1200-10; Vector Laboratories, Inc.) onto slides. They were then analyzed for Ki-67 staining.

Statistical analysis. A Student's t-test was used to compare data between two groups and for multiple group comparisons, one-way ANOVA and the Bonferroni post hoc test were applied. The differences were considered statisticaly significant at $P<0.05$. Data are expressed as the means ± SEM.

Results

Engineered toxin-resistant cells can express secretable and functional forms of targeted toxins. 293T cells were engineered for toxin resistance using single-stranded oligonucleotides designed to encode mutant elongation factor-2 (ssODN-mEF-2) as previously described by the authors (22) (Fig. S1A). Cell viability assays indicated toxin resistance (TR) in the 293T-ssODN (293 oligo) cells compared with the controls post-treatment with various concentrations of DT (Fig. S1B). The expression and secretion of targeted toxins was observed in the conditioned medium from toxin-resistant 293 oligo cells transduced with LVs bearing either ENb-PE or IL13-PE and the control (Fig. S1C-E). Subsequently, to examine the functionality of ENb-PE or IL13-PE on GBM cells, GBM lines expressing a luciferase reporter (destabilized luciferase; dsluc) were established. Treatment of GBM cells with IL13-PE and ENb-PE resulted in the blocking of protein synthesis and a dynamic change in gene expression was detected according to dsluc signal (Fig. S2A); significant changes were also detected in cell proliferation and viability (Fig. S2B-G). Subsequently, the EGFR and IL13Rα2 levels were assessed in a panel of GBM lines, including patient-derived cells (Fig. 1A) and the efficacy of IL13-PE and ENb-PE was evaluated on these cell lines. A direct association between EGFR/ IL13Rα2 expression and the response to ENb-PE/IL13-PE was observed in all GBM lines tested (Fig. 1A and B). Taken together, these results indicate that toxin-resistant 293oligo can be engineered to express IL13-PE and ENb-PE, which have a targeted therapeutic effect on GBM cells.

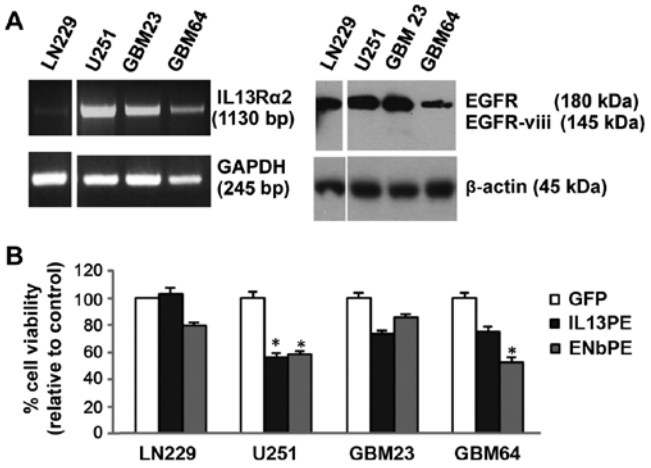

Figure 1. Therapeutic response to targeted toxins varies in TRAIL-resistant GBM cells. (A) RT-PCR and western blot analysis showing IL13Rα2 and EGFR expression levels in GBM cells. (B) Plot showing the viability of TRAIL resistant GBM cells post- treatment with targeted toxin (IL13-PE or ENb-PE) conditioned medium as compared to the GFP (control). Data represented as means ± SEM and P-values were determined by one-way ANOVA and a post hoc test (Bonferroni) (*$P<0.05$ vs. control). TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; GBM, glioblastoma; IL13Rα2, interleukin 13 receptor α2; EGFR, epidermal growth factor receptor.

Targeted toxins upregulate DR4/5 and induce a decrease in the expression of anti-apoptotic XIAP in resistant GBM cells. GBM cells (LN229, GBM4, GBM18, GBM23, GBM64 and BT74) exhibited varying degrees of TRAIL resistance (Fig. S3) and some exhibited significant resistance to targeted toxins (Fig. 1B). To evaluate the possibility of sensitizing the GBM cells the effects of targeted toxins to promote TRAIL-mediated, TRAIL-resistant wild-type LN229 (wt) and LN229 cells engineered to express IL13Rα2 (LN229-IL13Rα2) were treated with ENb-PE and IL13-PE targeting their cognate receptors EGFR and IL13Rα2, respectively (Fig. 2A). Western blot analysis revealed an increase in the total DR4/5 levels post-treatment with 25 ng/ml ENb-PE (Fig. 2B) and decreased anti-apoptotic XIAP levels in the LN229 cells, respectively (Fig. 2C). To examine the *in vitro* killing effect of the targeted toxin and TRAIL combination, a panel of TRAIL-resistant GBM cells were pre-treated with ENb-PE (25 ng/ml for 24 h) and these cells were then co-treated with 500 ng/ml TRAIL for 24 h. ENb-PE stimulated the TRAIL killing of highly resistant GBM lines with a significant decrease in cell viability ($P<0.001$ and $P<0.05$; Fig. 2D). For assessing the combined therapeutic efficacy of IL13-PE and TRAIL, a TRAIL-resistant line expressing IL13Rα2, LN229-IL13Rα2 was created (Fig. 2E). Similar to ENb-PE, western blot analysis revealed an increase in the total DR4/5 levels post-treatment with 25 ng/ml IL13-PE (Fig. 2F) and decreased anti-apoptotic XIAP levels (Fig. 2G) in LN229-IL13Rα2 cells treated with a combination of IL13-PE and TRAIL. This combined treatment resulted in a significant

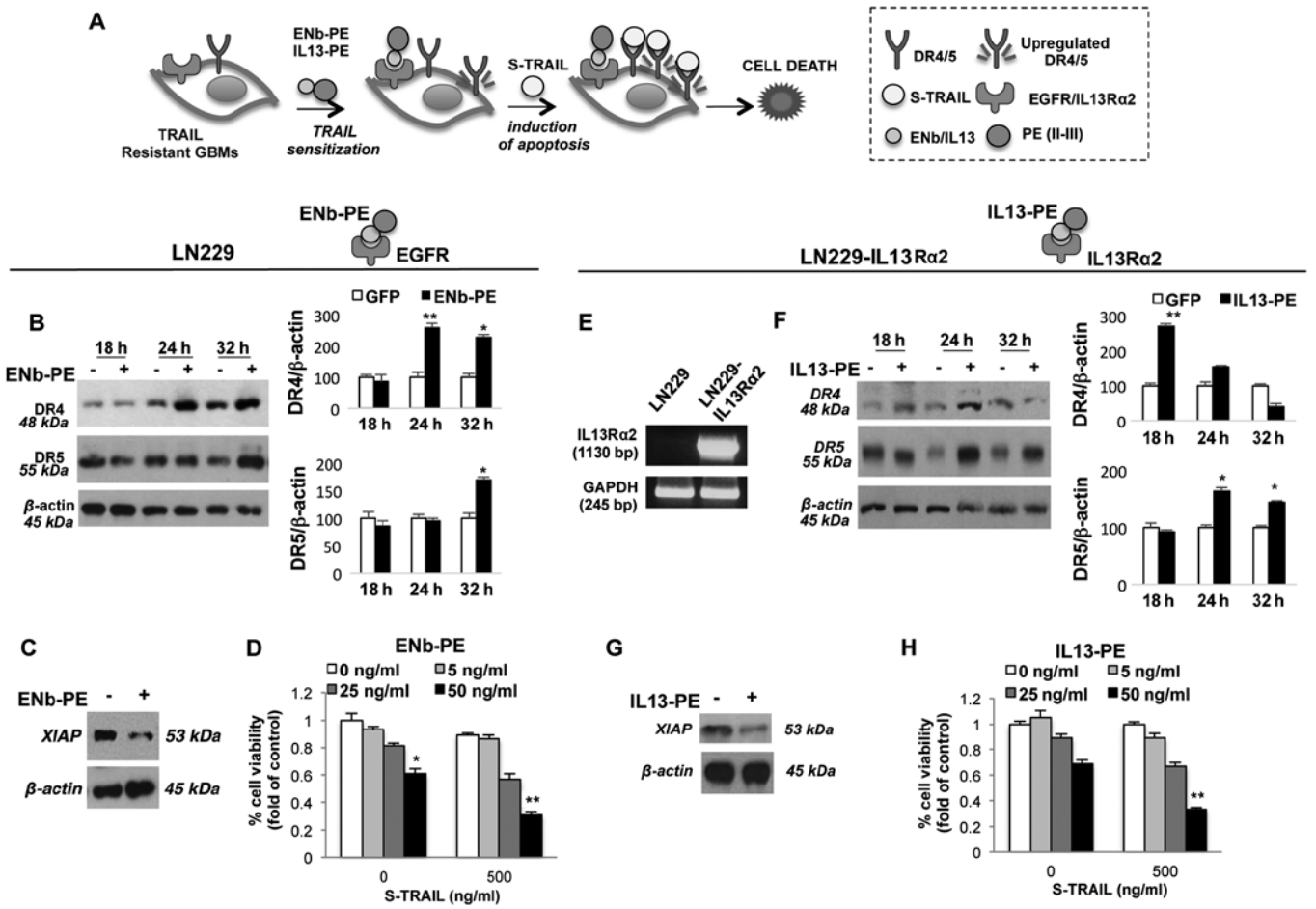

Figure 2. Targeted toxins upregulate DR4/5 and sensitize GBMs to TRAIL-mediated apoptosis. (A) Schematic demonstration of sensitization to TRAIL-mediated apoptosis upon IL13-PE/ENb-PE treatment in resistant GBM cells. (B) Western blot analysis of DR4/5 expression in LN229 cells treated with either ENb-PE (25 ng/ml) or the control for 18, 24 and 32 h. Representative plots showing the results of ImageJ analysis of band densities. (C) Western blot analysis of XIAP expression following treatment of LN229 cells with ENb-PE for 24 h. (D) Viability of LN229 cells pre-treated with 0-50 ng/ml ENb-PE for 24 h and subsequent treatment with 0-500 ng/ml S-TRAIL for 24 h. (E) RT-PCR analysis of IL13Rα2 transcripts in engineered LN229-IL13Rα2. (F) Western blot analysis of DR4/5 expression in LN229-IL13Rα2 cells treated with either IL13-PE (25 ng/ml) or control medium for 18, 24 and 32 h. Representative plots showing the results of ImageJ analysis of band densities. (G) Western blot analysis showing XIAP levels in LN229-IL13Rα2 cells treated with IL13-PE for 24 h. (H) Viability of LN229-IL13Rα2 cells pre-treated with 0-50 ng/ml IL13-PE and subsequent treatment with 0-500 ng/ml S-TRAIL for 24 h. Data represent the means of ± SEM and P-values were determined by an unpaired Student's t-test when comparing 2 groups. For multiple comparisons, one-way ANOVA and a post hoc test (Bonferroni) was applied (*P<0.05 and **P<0.001 compared to control). TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; GBM, glioblastoma; IL13Rα2, interleukin 13 receptor α2.

decrease in cell viability (P<0.001) in TRAIL-resistant LN229-IL13Rα2 GBM lines (Fig. 2H). Subsequently, the sensitizing effect of IL13-PE or ENb-PE treatment was assessed in a TRAIL semi-sensitive GBM line, U251, in real-time by DR4/5 promoter-luciferase imaging. As was expected, DR4/5 receptor expression levels were increased post-treatment with IL13-PE in the U251 cells, which were previously engineered with the construct bearing luciferase reporter under the DR4/5 promoter. IL13-PE/ENb-PE and TRAIL combination was much more potent than any mono-treatments on the semi-sensitive U251 cells. (Fig. S4A and C). These findings reveal that treatment with IL13-PE and ENb-PE results in the upregulation of DR4/5 and the downregulation of FLIP which then leads to the sensitization of the cells to TRAIL-mediated apoptosis.

Co-operation of targeted toxins with TRAIL kills GBM cells through both caspase-8- and caspase-9-mediated apoptosis. TRAIL-mediated apoptosis is a rapid process characterized by the activation of a cascade of intracellular proteases, or caspases and the cleavage of numerous intracellular proteins, resulting in cell death within 24-48 h post-TRAIL treatment (12). In the present study, to examine the apoptosis of GBM cells exposed to a combination of targeted toxins and TRAIL, since LN229 expresses EGFR and lacks IL13Rα2 transcripts, the therapeutic efficacy of ENb-PE was tested and the apoptosis of the LN229 cells was analyzed. To examine the efficacy of IL13-PE, LN229-IL13Rα2 cells were utilized, engineered as described above (Fig. 2E). The findings revealed that both toxin fusion proteins targeted their cognate receptors and sensitized the LN229 cells to TRAIL-mediated therapy. The activation of caspase-8-mediated apoptosis was triggered in TRAIL-resistant GBM cells when the cells were pre-treated with targeted toxins, while TRAIL mono-treatment did not lead to the cleavage of caspase-8 in any resistant lines. Similar results were observed for caspase-9 and poly ADP ribose polymerase (PARP) (Fig. 3A and B). A major contributor of TRAIL resistance is a caspase-8 analogue, FLIP, which is direct contact with DR4/5 and caspase-8 or -10 dynamics (10-12). Thus, the

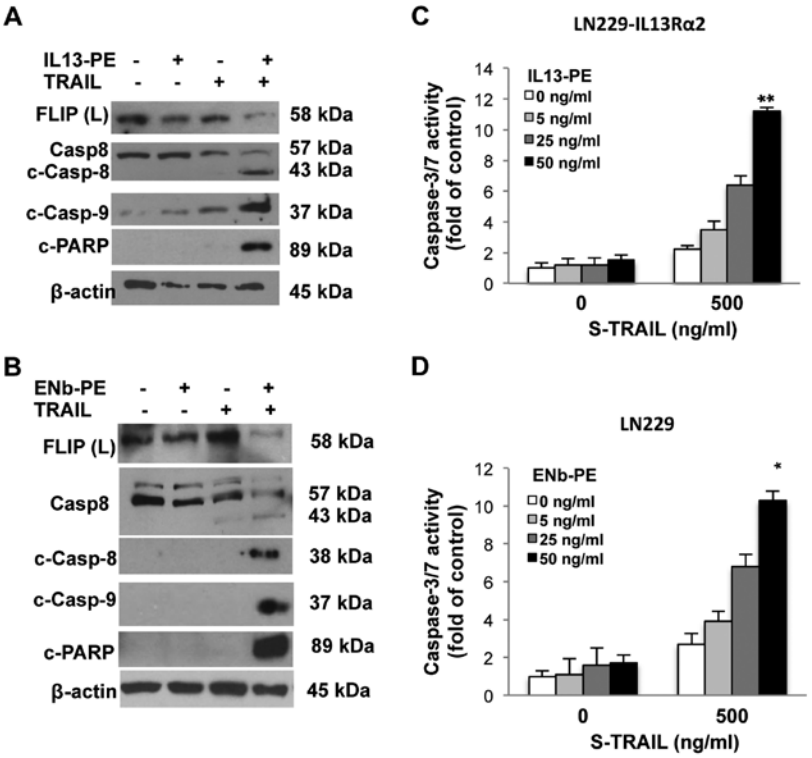

Figure 3. Resistant GBM cells undergo TRAIL-mediated apoptosis upon targeted toxin pre-treatment. (A and B) Western blot analysis of apoptosis in 25 ng/ml IL13-PE pre-treated LN229-IL13Rα2 cells and 25 ng/ml ENb-PE pre-treated LN229 cells followed by 24 h of incubation with 500 ng/ml S-TRAIL (control; GFP conditioned medium). Lysates were probed with antibodies against cleaved PARP, FLIP, caspase-8 and cleaved caspase-9 with β-actin control loadings. (C and D) Plots showing caspase-3/7 activity in LN229-IL13Rα2 and LN229 cells pre-treated with IL13-PE or ENb-PE, respectively for 24 h (0-50 ng/ml) and subsequently treated with 500 ng/ml S-TRAIL for 18 h. Data represent the means ± SEM and P-values were determined by one-way ANOVA and a post hoc test (Bonferroni) (*P<0.05 and **P<0.001 compared to control). TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; GBM, glioblastoma; IL13Rα2, interleukin 13 receptor α2; c-Casp, cleaved caspase.

present study evaluated the interaction of FLIP and caspase-8 cleavage in GBM cells upon treatment with the ENb-PE and TRAIL combinations. Western blot analysis revealed that caspase-8 was cleaved when the FLIP impediment was removed in GBM cells upon treatment with the targeted toxins and TRAIL in combination, but not with treatment with TRAIL or targeted toxins alone (Fig. 3A and B). In a more TRAIL sensitive line, U251, caspase-9 cleavage was detected with the TRAIL and IL13-PE/ ENb-PE combination, while cleaved caspase-8 was detected with both the TRAIL and TRAIL and IL13-PE/ENb-PE combination treatments. Furthermore, the findings for PARP cleavage were in accordance with those of caspase cleavage (Fig. S4B). In addition to the biochemical analysis of apoptosis, relative caspase-3/7 activation in GBM cells treated with increased concentrations of targeted toxins and TRAIL, was quantified. The data revealed an association between caspase-3/7 activation and GBM viability in a concentration-dependent manner (Figs. 3C and D, and S4D). To sum up, these results revealed that TRAIL combined with targeted toxins executed both caspase-8- and caspase-9-dependent apoptotic machinery, and a final GBM cell killing occurred in a synergistic manner, involving not only intrinsic, but also extrinsic apoptotic pathways.

Combination of IL13-PE/ENb-PE and TRAIL have therapeutic efficacy in patient-derived primary GBM lines. A number of studies have suggested that the majority of the chemo- and radiotherapy-resistant cell population of GBM arises from the CSC phenotype (35-38). In the present study, to investigate the therapeutic response of PE cytotoxins and TRAIL combination on $CD133^{+}$ glioma stem cells, GSCs (also known as glioma initiating cells), GSC lines that were previously developed and characterized from human GBM tissue were used as a xenograft model to examine several experimental therapeutics both *in vitro* and *in vivo*. These CSC enriched GSCs can grow in culture as spheres and when implanted intracerebrally into immunodeficient mice, they can form highly invasive and angiogenic tumors (34). The therapeutic efficacy of the IL13-PE/ENb-PE and TRAIL combination in highly TRAIL-resistant patient lines was associated with those established lines expressing both 2 cognate receptors. Either IL13-PE (25 ng/ml) or ENb-PE (25 ng/ml) with TRAIL (500 ng/ml) post-treatment induced apoptosis through the cleavage of caspase-8 and caspase-9 (Fig. 4A). Furthermore, IL13-PE/ENb-PE and TRAIL co-treatment of the GCSs resulted in significant cell death in resistant GSCs expressing both EGFR and IL13Rα2 (Fig. 4B and C). These findings revealed that TRAIL combination with targeted toxins induced the mechanism-based killing of TRAIL-resistant GSC lines in a similar manner with treated GBM cell lines (Fig. 5).

Discussion

The present study demonstrated a mechanism-based rationale for combining targeted PE cytotoxins with TRAIL to trigger

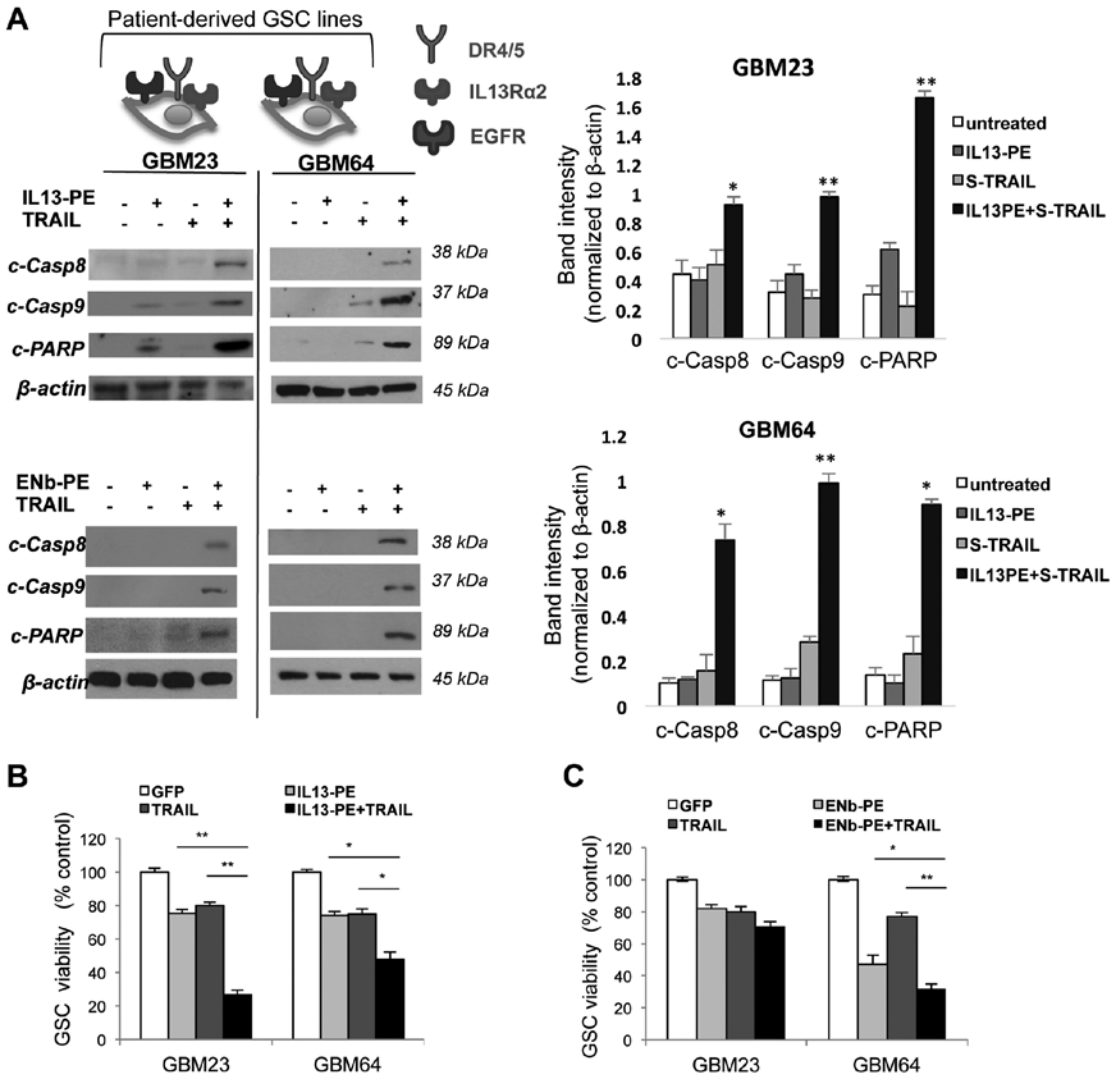

Figure 4. Combination of S-TRAIL with targeted toxins induces and enhances death signals in patient-derived GBM lines. (A) Western blot analysis of apoptosis in TRAIL-resistant patient-derived GBM lines (GBM23 and GBM64). Cells were treated with 25 ng/ml targeted toxins (either IL13-PE or ENb-PE) for 24 h then subsequently treated with 500 ng/ml S-TRAIL for 24 h. Lysates were probed with antibodies against cleaved PARP, caspase-8 and caspase-9 and β-actin. (B and C) Plots indicate the viability of both GBM23 and GBM64 cells pre-treated with either IL13-PE or ENb-PE (considering the cognate receptor expressions) for 24 h and subsequently with S-TRAIL for an additional 24 h. Data represent the means ± SEM and P-values were determined by one-way ANOVA and a post hoc test (Bonferroni) (*P<0.05 and **P<0.001). TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; GBM, glioblastoma; IL13Rα2, interleukin 13 receptor α2.

tumor-killing in resistant GBMs. Targeted toxins result in the upregulation of DR4/5 and the downregulation of FLIP and XIAP, leading to the sensitization of resistant GBMs to TRAIL-mediated apoptosis.

TRAIL is a novel pro-apoptotic cytokine targeting cancer cells, whilst sparing healthy cells. A number of TRAIL-based therapies have encountered difficulties in phase I/II clinical trials with no marked success for use in phase III trials. On the other hand, the authors, as well as other researchers have reported that GBMs display various degrees of response to TRAIL monotherapy, similar to a number of other cancer cells (16,17,39). There is therefore an urgent need to overcome TRAIL resistance with combination therapy approaches. To this end, a number of TRAIL sensitizers, as well as other anticancer agents targeting different death-inducing mechanisms are underway for the establishment of tumor regression and the prevention of tumor reoccurrence. Barriers to these approaches include the short half-life of therapeutic agents, blood-brain barrier characteristics, normal tissue toxicity and limited therapeutic effect caused by resistance mechanisms. In addition, current TRAIL sensitizers are synthetic compounds and have similar difficulties in therapeutic applications. In the present study, secretable targeted toxins were developed that function as a TRAIL sensitizers in GBMs. These findings have potential for development as a new methodology for GBM therapy, combining three advantages: i) Localized therapy ensures high concentration of the toxin at the tumor site; ii) breaking TRAIL resistance with mechanism-oriented sensitization; iii) maximizing residual tumor eradication using a clinically relevant GBM resection model.

In tumor cells with a low or no response to TRAIL, the cells can develop resistance to TRAIL via different mechanisms. Previously, it was demonstrated that GBM cells have varying levels of DR4/5 expression, which are the major determinants of TRAIL-mediated apoptosis (16,39). Resistance to TRAIL can occur either via the upregulation of the death receptors (DR4/5)

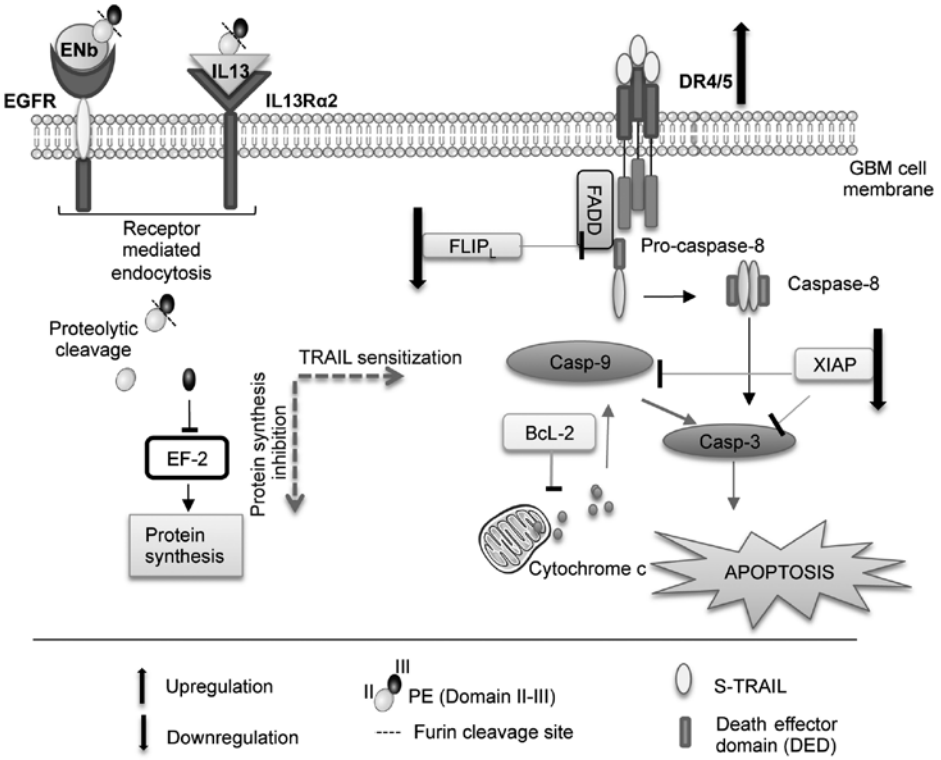

Figure 5. Schematic representation of intracellular signaling in targeted toxin- and TRAIL-treated GBM cells. Upon binding to GBM cell surface to the cognate receptor, targeted toxins (either IL13-PE or ENb-PE) are internalized through receptor mediated endocytosis (RME) and cleaved cytotoxic domain inactivates elongation factor-2 (EF-2) and inhibits protein synthesis at the translational level. Cellular dynamics mediated by targeted toxins then lead to the upregulation of TRAIL receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) and the downregulation of FLIP and XIAP anti-apoptotic proteins which are involved in death receptor-mediated apoptosis (FLIP) and mitochondrial apoptotic pathway respectively. As TRAIL binds to its receptors, DR4/5 upregulation and FLIP downregulation releases receptor death domain binding to procaspase-8 and active caspase-8 cleaves downstream caspases. Additionally, the decrease in XIAP levels induces caspase-9 activation, resulting in the triggering of apoptotic cascades. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; GBM, glioblastoma; IL13Rα2, interleukin 13 receptor α2; FLIP, FLICE-inhibitory protein; XIAP, X-linked inhibitor of apoptosis protein.

or the downregulation of anti-apoptotic proteins. cFLIP tends to bind the death domain of DR4/5 through the FADD adaptor protein and competes with procaspase-8, and therefore inhibits the activation of downstream apoptotic cascades. Decreased cFLIP levels can enhance signaling through caspase-8 and therefore the downstream caspases (11-17). The depletion of some anti-apoptotic proteins, such as XIAP, can trigger the intrinsic apoptotic pathway, which leads to the cleavage of caspase-9. As the present study detected both the upregulation of DR4/5 and the downregulation of FLIP and XIAP, it was considered that both mechanisms may play a role in the IL13-PE-mediated enhancement of TRAIL-induced apoptosis. This indicates that toxin and TRAIL treatments result in synergistic cell death involving the activation of both the extrinsic and intrinsic apoptotic pathways. Furthermore, a sensitizing agent that can upregulate DR4/5 expression potentially induces death signals through TRAIL-mediated apoptosis. Additionally, a sensitizing agent may also influence downstream effectors of the apoptotic cascade, such as FLIP, XIAP, Bcl-2 family proteins, and others that can lead to increased cancer cell death. In the present study, it was demonstrated that targeted toxins (either IL13-PE or ENb-PE) can sensitize even highly resistant cancer cells to TRAIL within 24 h and subsequently TRAIL can kill these cells in <24 h. As shown in Fig. S2, the PE-mediated inhibitory mechanism does not involve 100% translational inhibition within 24 h. This can be explained as follows: The PE toxin (domain III) interaction with EF-2 makes EF-2 dysfunctional, while untargeted EF-2 can still guide translation and be involved in maintaining protein synthesis, resulting in upregulation of some proteins. As such, DR4/5 is upregulated which then leads to more TRAIL binding and enhanced death signaling. Such protein dynamics may also explain why only approximately 50% of the protein synthesis inhibition occurs upon toxin treatment. This also requires certain time points to be chosen for TRAIL treatment post-PE treatment. The present study observed the upregulation of DR4/5 at approximately 24 h and therefore optimized the TRAIL treatment time point at 24 h post-PE treatment (Fig. 2B and F).

As the present study detected both the upregulation of DR4/5 and the downregulation of FLIP and XIAP, it was considered that both mechanisms may play a role in the IL-13PE/ENb-PE-mediated enhancement of TRAIL-induced apoptosis. This indicates that toxin and TRAIL treatments result in synergistic cell death involving the activation of both extrinsic and intrinsic apoptotic pathways. To our knowledge, this is the first study linking targeted toxin-mediated TRAIL sensitization with DR4/5 modulation. The findings presented herein shed new light on the mechanisms through which DR4/5 upregulation is regulated by targeted toxins. One possibility is that unfolded proteins released during toxin processing (protein synthesis inhibition at the translational level) within the cell may cause ER stress and this may then result in DR4/5 upregulation through reactive oxygen

species (ROS) activation. ROS are involved in the upstream modulation of TRAIL signaling, which leads to an enhanced TRAIL sensitivity in various cancer cells by inducing the expression of death receptors (40). Alternatively, the mechanism can be related to the modulation of transcription factors which then drives DR4/5 gene expression. Further studies are required to clarify the DR4/5 upregulation mechanism in IL13-PE/ENb-PE-targeted toxin-treated cancer cells. Moreover, it was demonstrated that GBM treatment with targeted toxins downregulated $FLIP_L$, resulting in the activation of caspase-8-mediated apoptosis, since FLIP acts as a caspase-8/10 inhibitor and eventually an intracellular blocker of the apoptotic TRAIL signal. Downstream of the apoptotic cascade, toxin-mediated therapy decreases XIAP levels, which releases the blockage of caspase-3 and caspase-9; hence, it enhances intracellular death signals. As a proof of concept, wild-type TRAIL-resistant LN229 (LN229-wt) cells were engineered to overexpress IL13Rα2. The cells were then treated with IL13-PE and TRAIL to evaluate TRAIL sensitization upon cognate receptor expression. The results were in accordance with the current findings on ENb-PE-treated LN229 cells. ENb-PE-treated LN229 and IL13-PE pre-treatment of LN229-IL13Rα2 cells upregulated DR4/5, depleted FLIP and XIAP, and resulted in significant cell death when combined with TRAIL. In line with this finding, LN229-wt cells did not exhibit any significant decrease in viability upon treatment with neither S-TRAIL alone nor IL13-PE and S-TRAIL, since LN229-wt lacks the cognate IL13Rα2 receptor and is highly resistant to TRAIL. These results reveal that toxin-mediated TRAIL sensitization might occur in any resistant GBMs with cognate receptor expression.

Moreover, TRAIL semi-sensitive GBMs undergo superior cell death by the combination of TRAIL with targeted toxins. According to the current findings, in GBMs, caspase-8-dependent apoptosis occurred induced by TRAIL. Caspase-9-mediated apoptosis also occurred due to the enhancement of death-inducing signals; the cleaved form of caspase-9 was detected in only the toxin- and TRAIL-treated groups suggesting that a subpopulation of cancer cells (in terms of TRAIL response) in semi-sensitive GBMs exhibit TRAIL resistance. In these cells, caspase-9-mediated apoptosis is most likely induced in the presence of targeted toxins. Therefore, in addition to the attenuation of anti-apoptotic proteins, crosstalk between extrinsic and intrinsic apoptosis pathways may exist explaining toxin and TRAIL co-operation. On the other hand, targeted toxins can act as inhibitors of protein synthesis and lead to cell death when used alone. The key point is that the PE toxins alone lead to cell cycle arrest at 24 h and the killing effect is measured in the following 48 h. For this reason, 24 h pre-treatment with PE toxins is most likely sufficient to modulate protein levels affected by synthesis inhibition, which then results in DR4/5 upregulation and depletion of anti-apoptotic proteins. To summarize, in TRAIL-resistant GBMs, targeted toxins contribute to cancer cell death via two dynamic mechanisms: i) TRAIL sensitization in the first 24 h and subsequent TRAIL-mediated apoptosis; ii) toxin-mediated killing via protein synthesis blockage. To sum up, these two modes of toxin action can enable the selective killing of GBMs in a synergistic manner using the specific advantages of TRAIL and targeted PE toxin engagement.

Since GBM cells-similar to a number of other cancer cells-exhibit some level of resistance to both TRAIL and PE cytotoxins, the selective killing of primary GBMs with a combination of targeted toxins and TRAIL is a favorable approach as the combination induces cell death via different mechanisms. To highlight the clinical potential of this strategy and considering the heterogeneity of patient-derived GSCs to TRAIL therapy, the present study analyzed toxin-directed TRAIL sensitization in a panel of primary GBMs. The data demonstrated that both IL13-PE and ENb-PE cytotoxins overcame TRAIL resistance and with TRAIL involvement, greater cell death was achieved in primary GBM23 and GBM64 cells expressing target receptors.

To the best of our knowledge, this is the first report of TRAIL sensitization via the upregulation of DR4/5 by targeted toxins. Since toxin and TRAIL co-operation leads to the orchestrated killing of GBM cells, it will be of great interest to utilize the same strategy for other malignancies, which are difficult to treat with TRAIL monotherapy. Furthermore, the biochemically-proven TRAIL sensitization effects of PE in patient-derived GSC lines reveal the clinical importance of the current findings and provides a novel strategy for GBM treatment modalities.

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Availability of data and materials

All the materials used and the data generated are included in the present manuscript or are available from the corresponding author upon reasonable request.

Authors' contributions

NK designed the study, and was responsible for the provision of the study material, collection and assembly of data, data analysis and interpretation, manuscript writing, revision and the final approval of the manuscript. DS designed the study, and was involved in the collection and assembly of data, data analysis and interpretation, manuscript writing, and the final approval of the manuscript. ERL was involved in the provision of the study material, collection and assembly of data, data analysis and interpretation, and the final approval of manuscript. KS was involved in the conception and design of

the study, and in the provision of study material, data analysis and interpretation, manuscript writing, revision and the final approval of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study used patient derived established GSC (glioma stem cell) lines, the use of which was approved by the Massachusetts General Hospital (MGH) Ethics Committee.

Patient consent for publication

Not applicable.

Competing interests

KS owns equity in and is a member of the Board of Directors of AMASA Therapeutics, a company developing stem cell-based therapies for cancer. The interests of KS were reviewed and are managed by Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict of interest policies. The authors declare no competing financial interests.

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