Exceptionally potent anti-tumour bystander activity of an scFv:sTRAIL fusion protein with specificity for EGP2 towards target antigen-negative tumour cells.

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Abstract
Previously, we reported on the target cell-restricted fratricide apoptotic activity of scFvC54:sTRAIL, a fusion protein comprising human soluble TRAIL genetically linked to the antibody fragment scFvC54 specific for the cell surface target antigen EGP2. In the present study, we report that the selective binding of scFvC54:sTRAIL to EGP2-positive target cells conveys an exceptionally potent pro-apoptotic effect towards neighbouring tumour cells that are devoid of EGP2 expression (bystander cells). The anti-tumour bystander activity of scFvC54:sTRAIL was detectable at target- to bystander cell ratios as low as 1:100. Treatment in the presence of EGP2-blocking or TRAIL-neutralizing antibody strongly inhibited apoptosis in both target and bystander tumour cells. In the absence of target cells, bystander cell apoptosis induction was abrogated. The bystander apoptotic activity of scFvC54:sTRAIL did not require internalization, enzymatic conversion, diffusion, or communication between target and bystander cells. Furthermore, scFvC54:sTRAIL showed no detectable signs of innocent bystander activity towards freshly isolated blood cells. Further development of this new principle is warranted for approaches where cancer cells can escape from antibody-based therapy due to partial loss of target antigen expression.

Introduction
In recent years several antibody-based therapies that target tumour-associated membrane antigens have entered clinical trials with promising results\(^1\)-\(^3\). However, curative treatment is frequently not achieved due to therapy-resistant recurrences emerging after initial rounds of seemingly successful treatment\(^4\)-\(^6\). It has been shown that within one tumour mass different stages of malignant progression and various oncogenic mutations can occur simultaneously, leading to the development of heterogeneous tumour cell phenotypes\(^7\)-\(^12\). Heterogeneous and lost target antigen expression are likely to be responsible for many of the therapeutic failures observed in current antibody-based therapies\(^4\)-\(^6\).

Therefore, strategies have been developed to take advantage of the so-called ‘bystander effect’, which aims to eliminate tumour cells with reduced or lost target antigen expression. The bystander effect is based on the principle that targeted tumour cells are not only eliminated, but are also exploited to convey the therapeutic effect towards neighbouring tumour cells devoid of target antigen expression. Bystander effects have been described for several antibody-based therapeutic approaches\(^13\),\(^14\), and more recently

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for gene therapy using FASL and TRAIL, two members of the Tumour Necrosis Family of death inducing ligands\textsuperscript{15-18}.

TRAIL is of particular interest for its tumour-restricted apoptosis inducing capacity in a wide range of neoplastic cells while sparing normal tissues. TRAIL is expressed as a type II transmembrane protein (memTRAIL)\textsuperscript{19,20} on a broad spectrum of tissues ranging from peripheral blood lymphocytes, spleen, and thymocytes to many solid organs but is absent in brain, liver, and testis.

A unique TRAIL receptor system has been uncovered in which the distinct receptors TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, and osteoprotegerin (OPG), can differentially bind and interact with TRAIL. After ligation, TRAIL-R1 and TRAIL-R2 recruit the intracellular FAS-associated Death Domain adapter protein (FADD) and the initiator caspase-8 or -10, thereby forming the Death Inducing Signalling Complex (DISC)\textsuperscript{21-28}. Assembly of the DISC results in activation of caspase-8 or -10 that subsequently cleave and activate effector caspases, such as caspase-3, -6, and -7, leading to e.g. PARP cleavage and ultimately apoptotic cell death. TRAIL-R3, TRAIL-R4, and OPG lack (functional) death domains and after ligation, do not induce apoptosis.

TRAIL-R1 and TRAIL-R2 have a broad and partly overlapping pattern of expression, suggesting that they may serve as an alternate or ‘backup’ system, allowing the immune system to control aberrant cells even if one of the receptors has failed. Recently, it was shown that TRAIL-R1 and TRAIL-R2 have rather distinct crosslinking requirements for the initiation of apoptosis\textsuperscript{29}. Both recombinant soluble TRAIL (sTRAIL), consisting of the extra-cellular domain of TRAIL, and memTRAIL can efficiently activate TRAIL-R1 even at low concentrations, whereas TRAIL-R2 can only be activated by memTRAIL or sTRAIL that is secondarily crosslinked by antibodies.

To date, various forms of recombinant soluble TRAIL have been generated of which potent anti-tumour activity has been demonstrated in several xenograft mouse models of human cancers, including colorectal cancer\textsuperscript{30,31}, glioblastoma\textsuperscript{31}, and breast cancer\textsuperscript{32}. These sTRAIL preparations have retained the selective apoptotic activity towards transformed cells, but lack an intrinsic targeting capacity that allows for preferential binding to TRAIL receptors expressed on tumour cells. Moreover, sTRAIL is not very effective in signalling apoptosis in tumour cells that predominantly express TRAIL-R2.

It has been shown that crosslinking of agonistic TRAIL receptors is required to efficiently obtain cell death. In a previous report, Wajant et al.\textsuperscript{33} demonstrated that signalling capacity of sTRAIL for TRAIL-R2 could be restored by genetic fusion to a recombinant antibody fragment (scFv) recognizing the tumour stroma marker fibroblast activation protein
(FAP). Independently, we developed a TRAIL fusion protein, designated scFvC54:sTRAIL, in which the human scFv antibody fragment C54 is genetically linked to the N-terminus of human soluble TRAIL. The high affinity scFvC54 antibody domain specifically targets EGP2 (also known as epithelial cell adhesion molecule (Ep-CAM), or CO17-1a antigen), an established cell surface target antigen over-expressed on a variety of carcinomas. Selective binding to EGP2 results in accretion of scFvC54:sTRAIL at the cell surface of targeted cells only, converting soluble scFvC54:sTRAIL into a membrane bound form of TRAIL. Subsequently, a surplus of sTRAIL domains displayed on the target cell surface is available for the crosslinking of TRAIL-R2 on neighbouring tumour cells, resulting in efficient and target antigen-restricted reciprocal fratricide apoptosis induction.

In the present study, we analyzed whether targeting of scFvC54:sTRAIL to EGP2-positive cells can be used to convey a pro-apoptotic bystander effect towards neighbouring tumour cells devoid of EGP2 expression, as schematically depicted in Fig.1. Interestingly, we observed an exceptionally potent bystander apoptotic effect of scFvC54:sTRAIL, which critically depended on the presence of EGP2-positive target cells. Bystander apoptosis induction by scFvC54:sTRAIL might be applicable for the treatment of human cancer cells that escape current antibody-based therapy due to partial loss of target antigen expression.

**Fig.1. Target cell and bystander cell apoptosis induction by scFvC54:sTRAIL.** Binding of scFvC54:sTRAIL to the abundantly expressed target antigen EGP2 (▲) results in immobilization of scFvC54:sTRAIL at the cell surface of EGP2-positive cells only. Subsequently, membrane bound scFvC54:sTRAIL induces fratricide apoptosis by reciprocal crosslinking of TRAIL-R1/R2 (□) on neighboring EGP2-positive target cells. Analogously, immobilized scFvC54:sTRAIL on target cells can induce crosslinking of agonistic TRAIL receptors on the cell surface of a neighboring tumor cell devoid of EGP2 expression, resulting in apoptosis induction of one or more bystander cells (diagram is not to scale).
Materials & Methods

Monoclonal antibodies and scFv antibody fragment

Monoclonal antibody (MAb) MOC31 is a murine IgG1 with high affinity specificity for human EGP2\(^{35}\). MAb MOC31 was directly labelled with phyco-erythrin (PE), yielding MOC31-PE using standard procedures. The anti-EGP2 scFvC54 (kindly provided by Prof. T. Logtenberg, Utrecht University, The Netherlands), has been previously selected from a large semi-synthetic phage display library with random human VH-VL pairings and has a VH(G4S)3-VL format\(^{36}\). MAb MOC31 and scFvC54 compete for binding to the same epitope on the extracellular domain of EGP2. A multimeric form of the extracellular domain of EGP2 (sEGP2) was produced and purified as described previously\(^{37}\). Where indicated, multimeric sEGP2 was used to secondarily crosslink scFvC54:sTRAIL. TRAIL-neutralizing MAb 2E5 was purchased from Alexis (Kordia Life Sciences, Leiden, The Netherlands). MAb 2E5 neutralizes TRAIL activity by binding to an epitope on the extracellular domain of TRAIL that inhibits binding to the various TRAIL receptors.

Cell lines and EGP2 transfectants

Human cell lines Jurkat (acute lymphoblastic T cell leukemia), Ramos (B cell lymphoma), and U87MG (glioblastoma), all of which are EGP2-negative, were purchased from the ATCC. EGP2-positive variants of the above cell lines were generated by retroviral transduction. In short, EGP2 cDNA was cloned into a retroviral vector derivative of LZRS-pBMN-lacZ\(^{38}\) (kindly provided by Dr. G. Nolan, Stanford University School of Medicine, San Francisco, USA), yielding LZRS-EGP2-IRES-EGFP. To produce retroviral particles, LZRS-EGP2-IRES-EGFP was transfected into the amphotrophic packaging cell line Phoenix, using Fugene-6 transfection reagent according to manufacturer’s recommendations (Roche Diagnostics, Almere, The Netherlands). Transfected cells were selected by culturing in the presence of 1 \(\mu\)g/ml puromycin, 300 \(\mu\)g/ml hygromycin, and 1 \(\mu\)g/ml diphtheria toxin (BD Biosciences Clontech, Palo Alto, USA). Viral particle-containing supernatant was harvested after 3 days and used to transduce Jurkat, Ramos and U87MG cells. After overnight incubation, viral particle-containing supernatant was replaced by fresh medium. Transduced cells were subsequently sorted for simultaneous EGFP-fluorescence and EGP2 expression as detected by MOC31-PE using the MoFlo high-speed cell sorter (Cytomation, Fort Collins, USA). Analogous methods were employed to generate c-FLIP\(_{L}\) encoding retroviral particles, which were used to transduce Ramos cells. Ectopic over-expression of c-FLIP\(_{L}\) in Ramos.c-FLIP\(_{L}\) transduced cells was confirmed by immunoblotting of intracellular protein extracts. All cell lines were cultured at 37ºC in humidified 5% CO\(_2\) atmosphere.
Suspension cell lines (Jurkat, Jurkat.EGP2, Ramos, Ramos.EGP2, Ramos.c-FLIP,) were cultured in RPMI (Cambrex, New Jersey, USA) supplemented with 15% foetal calf serum (FCS). Adherent cell lines (U87MG, U87MG.EGP2) were cultured in DMEM (Cambrex) supplemented with 10% FCS.

Expression of TRAIL receptors
Membrane expression levels of TRAIL-receptors 1, 2, 3, and 4, were analyzed by flow cytometry using a TRAIL-receptor antibody kit purchased from Alexis. Briefly, cells were harvested, washed using serum free RPMI, and resuspended in 100 $\mu$l fresh medium containing the appropriate primary MAb. Specific binding of the primary antibody was detected using a PE-conjugated secondary antibody (DAKO, Glostrup, Denmark). All antibody incubations were performed at 0°C for 45 min and were followed by two washes with serum free medium.

Production of scFvC54:sTRAIL
The fusion protein scFvC54:sTRAIL, comprising the scFvC54 targeting domain, an intra-chain linker, and the sTRAIL effector domain, was produced in Chinese Hamster Ovary (CHO-K1) cells as previously described\textsuperscript{34}. Briefly, the expression plasmid pEE14scFvC54:sTRAIL was transfected to CHO-K1 cells, after which cells were selected for amplified medium secretion of the fusion protein using the glutamine synthetase method as described before\textsuperscript{39}. Single cell sorting of transfectants using the MoFlo high speed cell sorter (Cytomation) identified CHO-K1 clone 70C1 that stably secreted 3.44 $\mu$g/ml scFvC54:sTRAIL into the culture medium. Using the same procedures, Mock-scFvH22:sTRAIL, directed at the antigen CD64, not present on the cell lines used in this study, was generated and added in experiments where indicated.

Apoptosis induction assessed by viability assay
Where indicated, apoptosis induction apparent from loss of tumour cell viability was assessed by MTS assay (Promega Benelux b.v., Leiden, The Netherlands). Briefly, cells were seeded in flat bottom 96-well micro culture plates at a density of 3·10\textsuperscript{4} cells/well in 100 $\mu$l medium. After overnight culture, medium was replaced with fresh medium containing the various experimental conditions. After 16 h, MTS assay was performed according to manufacturer’s recommendations. Experimental apoptosis induction was quantified as the percentage apoptosis compared to medium control, which was set at 0% apoptosis. Each experimental and control group consisted of six independent wells.
Apoptosis induction assessed by loss of Mitochondrial Membrane Potential (ΔΨ)
Where indicated, apoptosis induction apparent from loss of ΔΨ was analyzed using the cell-permeant green-fluorescent lipophilic dye DiOC6 (Molecular Probes, Eugene, USA) as previously described. In short, after 16 h treatment cells were harvested by centrifugation (300xg; 5 min) and incubated for 30 min at 37°C with fresh medium containing 0.1 µM DiOC6, washed twice with phosphate buffered saline (PBS), and analyzed using flow cytometry.

Immunoblot analysis of caspase activation and PARP cleavage
Where indicated, apoptosis induction apparent from caspase-8 and caspase-3 activation and PARP degradation was assessed by immunoblot analysis using antibodies against active caspase-8 (Cell signalling technology, Beverly, MA, USA), active caspase-3 (BD biosciences, San Jose, CA, USA), and PARP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) respectively. Briefly, cells were seeded in 6 well plates at a final concentration of 0.5-10^6 cells/ml and treated as indicated. Cells were harvested by centrifugation (2,000xg; 10 min), lysed in lysis buffer (20 mM Tris-HCl, 5.0 mM EDTA, 2.0 mM EGTA, 100 mM NaCl, 0.05% SDS, 0.50% NP-40, 1 mM PMSF, 10 µg/ml Aprotinin, 10 µg/ml Leupeptin, pH 6.8), and sonicated on ice for 2x5 sec. Cleared supernatants were collected after centrifugation (15,000xg; 10min) and protein concentration was determined using Bradford method according to manufacturer's instructions (BioRad, Hercules, CA, USA). Samples were diluted 1:1 in standard SDS/PAGE-loading buffer containing 2-mercapto-ethanol and boiled for 10 minutes. Samples of 30 µg total protein were loaded and separated using 10% acryl amide SDS-PAGE, followed by electroblot transfer to nitrocellulose. Blots were incubated with the respective primary MAb and appropriate HRPO-conjugated secondary antibodies. Specific binding of MAb was detected using ECL (Roche diagnostics, Indianapolis, IN, USA). All antibody incubations were performed at room temperature for 1.5 h in PBS containing 5% bovine serum albumin, followed by 3 washes with PBS containing 0.1% Tween-20.

Distinctive fluorescent labelling of target and bystander cells
Differential cell membrane labelling of target and bystander cells was achieved using the Vybrant Multicolor Cell-Labelling kit (Molecular probes). EGP2-negative bystander cells were labelled with the red fluorescent dye DiI, while the corresponding EGP2-positive target cells were not labelled. Briefly, labelling was performed by incubation of bystander...
cells (1·10^6 cells/ml in serum free medium) with 5 µM DiI (37°C; 5 min) followed by three subsequent washes with medium (1,200xg; 5 min). Pellet was resuspended in medium whereupon the DiI-labelled bystander cells were mixed with non-labelled target cells at the target- to bystander ratios indicated at a final concentration of 0.5·10^6 cells/ml. Differential membrane fluorescent characteristics allow the target and bystander cells to be separately evaluated in mixed culture after treatment.

**Distinctive quantification of apoptosis in target and bystander cells by ΔΨ**

Non-labelled target cells and DiI-labelled bystander cells were mixed, at the indicated ratios, at a final concentration of 0.5·10^6 cells/well in a 12-well plate. After overnight culture, cell mixtures were treated with scFvC54:sTRAIL (300 ng/ml) for 16 h in the presence or absence of MAb MOC31 (5 µg/ml), caspase 8 inhibitor Z-IETD-FMK (1 µg/ml) (Calbiochem, San Diego, CA, USA), or TRAIL-neutralizing MAb 2E5 (1 µg/ml). The differential fluorescent characteristics of target and bystander cells were subsequently used to separately evaluate the amount of apoptosis induced in target and bystander cells by measuring ΔΨ with the fluorescent dye DiOC6 as described above.

**FACS-sorting of target and bystander cells after treatment**

FACS-sorting was applied to separate mixed target cells and bystander cells after treatment with scFvC54:sTRAIL. To this end, DiI-labelled bystander cells (Jurkat) were mixed with an equal amount of unlabelled target cells (Jurkat.EGP2) at a final concentration of 0.5·10^6 cells/ml. This mixed cell culture was treated with scFvC54:sTRAIL (300 ng/ml) for 6 h in the presence or absence of MAbs MOC31 (5 µg/ml) or 2E5 (1 µg/ml). After treatment, cell mixtures were collected and washed twice in fresh medium pre-cooled at 0°C. Subsequently, 2.5·10^6 cells of both the target and bystander cells were sorted using the MoFlo high-speed cell sorter. The sorted cells were found to be >99% pure and were separately analyzed for apoptotic features by immunoblot as described above.

**Fluorescence microscopy of bystander apoptosis induction**

Fluorescent microscopy was used to visualize bystander apoptosis induction in the adherent growing glioblastoma cell line U87MG. U87MG.EGP2 target cells, brightly expressing EGFP, were mixed at a 1:4 ratio with U87MG bystander cells at a final concentration of 0.5·10^6 cells/well on Lab-Tek chamber slides (Nalge Nunc int., Naperville, IL, USA). After overnight culture, spent medium was carefully aspirated and the mixed cell culture was subjected to treatment with scFvC54:sTRAIL (300 ng/ml) for 16 h, in the presence or
absence of MAb MOC31 (5 µg/ml) or MAb 2E5 (1 µg/ml), respectively. After treatment, apoptosis induction apparent from nuclear morphology was analyzed using the DNA binding dye Hoechst 33342 (Molecular probes). Both nuclear morphology and EGFP-fluorescence were visualized using a Quantimed 600S fluorescence microscope (Leica Camera Ag, Solms, Germany).

Quantification of innocent bystander apoptosis in isolated PBMCs
Leukocytes were isolated from EDTA anti-coagulated blood of healthy donors using the Ammonium Chloride method according to standard procedure. Briefly, blood was diluted 8-fold with cold Ammonium Chloride buffer and incubated for 10 min at 0°C, allowing the lysis of red blood cells. Subsequently, leukocytes were collected by centrifugation (300xg; 5 min). The above-described procedure was repeated to ensure complete lysis of all red blood cells. Isolated leukocytes were resuspended (RPMI, 10% human pool serum) and mixed at a target- to bystander ratio of 1:1 with target Jurkat.EGP2 cells that were labelled with the green fluorescent dye DiO (Molecular Probes). Mixed cultures were treated for 16 h with scFvC54:sTRAIL in the presence or absence of MAb MOC31 or MAb 2E5. The degree of apoptosis induction after treatment was analyzed by addition of the fluorescent DNA-binding dye Propidium Iodide (PI), and quantification of the percentage of PI-positive cells using flow cytometry.

Results

TRAIL-R expression in EGP2-transduced cell lines
Flow cytometric analysis of the retrovirally transduced cell lines Jurkat.EGP2, Ramos.EGP2, and U87MG.EGP2 revealed a strong homogeneous cell surface expression of EGP2 and intracellular EGFP fluorescence (data not shown). No significant differences in TRAIL receptor expression patterns were found between parental and EGP2-transduced cell lines (Table 1).

Target cell-restricted apoptosis induction by scFvC54:sTRAIL
EGP2-negative bystander cells (Jurkat, Ramos, U87MG) were not susceptible to apoptosis induction by scFvC54:sTRAIL. After prolonged treatment (16 h) with 300 ng/ml scFvC54:sTRAIL, cell cultures contained only low percentages of apoptotic cells (Fig.2A; 5%, 10%, and 3%, for Jurkat, Ramos, and U87MG, respectively). When treatment was performed in the presence of multimeric soluble EGP2 (3.5 µg/ml), which secondarily crosslinks scFvC54:sTRAIL, a strong increase in the percentages of apoptotic cells was
Table 1. Flow cytometric analysis of TRAIL receptor expression on target and bystander cells. EGP2-positive target cells and EGP2-negative bystander cells were analyzed for TRAIL receptor expression. Expression of TRAIL receptors was classified as not detectable (n.d.) when MFI was below 5 and subsequently in the following categories: 5<MFI<25 (*), 25<MFI<50 (†), 50<MFI<125 (‡).

<table>
<thead>
<tr>
<th>Cell Line</th>
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<td>Ramos</td>
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<td>Ramos.EGP2</td>
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<td>Jurkat</td>
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<td>U87MG</td>
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<td>U87MG.EGP2</td>
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Observed (92%, 84%, and 70% for Jurkat, Ramos and U87MG, respectively). Treatment of EGP2-positive target cells (Jurkat.EGP2, Ramos.EGP2, and U87MG.EGP2) with 300 ng/ml scFvC54:sTRAIL induced a strong increase in percentage of apoptotic cells (Fig.2B; 71%, 67%, 65%, respectively). Treatment of these EGP2-positive cells in the presence of EGP2-blocking MAb MOC31 strongly inhibited apoptosis induction (16%, 13%, and 5%, for Jurkat.EGP2, Ramos.EGP2 and U87MG.EGP2, respectively). Interestingly, although levels of TRAIL receptor expression were comparable for both parental and EGP2-transduced cell lines, the sensitivity to apoptosis induction by scFvC54:sTRAIL was somewhat reduced in the EGP2-transduced cell lines. Reduced sensitivity of EGP2-transduced cells may be related to the retroviral transduction procedure of these cell lines or to the ectopic overexpression of EGP2.

Distinctive quantification of apoptosis induction in target and bystander cells

When mixed cultures of Jurkat.EGP2 target cells and Jurkat bystander cells were treated with scFvC54:sTRAIL, strong apoptosis induction was observed in Jurkat.EGP2 target cells ranging from 55% at target to bystander ratio 7:3 to 20% at ratio 1:100 (Fig.3A). Apoptosis induction in Jurkat bystander cells ranged from 80% at ratio 7:3 to 17% at the remarkably low ratio 1:100 (Fig.3B). Treatment of bystander cells alone resulted in marginal induction of apoptosis (7%). Percentages of apoptotic cells were strongly reduced in both target and bystander cell populations when treatment was performed in the presence of EGP2-blocking MAb MOC31 (Fig.3A and B). Analogously, treatment with scFvC54:sTRAIL in the presence of TRAIL neutralizing MAb 2E5 completely abrogated apoptosis (Fig.3A and B). Furthermore, when mixed cultures of target and bystander cells were treated with the Mock-scFvH22:sTRAIL fusion protein, containing the antibody fragment domain scFvH22 of irrelevant specificity, no apoptosis induction was found in
Fig. 2. Target cell-restricted apoptosis induction by scFvC54:sTRAIL. **A:** EGP2-negative bystander cells (Jurkat, Ramos and U87MG) were treated with scFvC54:sTRAIL alone to determine sensitivity to apoptosis induction by non-crosslinked scFvC54:sTRAIL. Additionally, cells were treated with scFvC54:sTRAIL in the presence of multimeric sEGP2, which secondarily crosslinks scFvC54:sTRAIL, to determine intrinsic sensitivity to crosslinked scFvC54:sTRAIL. **B:** EGP2-positive target cells (Jurkat.EGP2, Ramos.EGP2, and U87MG.EGP2) were treated with scFvC54:sTRAIL for 16 h in the presence or absence of target antigen-competing MAb MOC31. Apoptosis induction was assessed by MTS assay. All values indicated in the graphs are the mean ± standard error of the mean of four independent experiments.

Jurkat.EGP2 target or Jurkat bystander cells (Fig. 3C). Similar experiments with mixed cultures of Ramos.EGP2 and Ramos, and the adherent cell lines U87MG.EGP2 and U87MG, further confirmed that treatment with scFvC54:sTRAIL potently induced both target and bystander apoptosis, whereas treatment with Mock-scFvH22:sTRAIL did not lead to significant apoptosis induction in target or bystander cells (Fig. 3C).

**Target and bystander apoptosis induction is caspase-8 dependent**

In a mixed culture of Ramos.EGP2 target cells and Ramos bystander cells (ratio 2:3), treatment with scFvC54:sTRAIL resulted in bystander apoptosis induction up to 65% (Fig. 4A). Treatment in the presence of the specific caspase-8 inhibitor Z-IETD-FMK strongly inhibited apoptosis induction in both target and bystander cells (Fig. 4A; 15% and 17%, respectively). Moreover, treatment of a mixed culture of Ramos.EGP2 target cells and bystander cells ectopically overexpressing the caspase-8 inhibitor c-FLIP<sub>L</sub> (Ramos.c-FLIP<sub>L</sub>), revealed that Ramos.c-FLIP<sub>L</sub> bystander cells were largely resistant to the pro-apoptotic bystander effect of scFvC54:sTRAIL (Fig. 4B; 17%). The residual induction of apoptosis in Ramos.c-FLIP<sub>L</sub> bystander cells was specifically inhibited by co-incubation of cells with MAb MOC31.
Immunoblot analysis of FACS-sorted target and bystander cells

Post-treatment sorting of a mixed culture of Jurkat.EGP2 target and Jurkat bystander cells (ratio 1:1), allowed for the separate evaluation of apoptotic features in target and bystander cells. Treatment with scFvC54:sTRAIL induced a clear activation of caspase-8 and caspase-3 in both Jurkat.EGP2 target cells (Fig.5A, lane 2) and Jurkat bystander cells (Fig.5B, lane 2). Activation of caspase-3 was accompanied by cleavage of its target.
Fig. 4. Target cell and bystander cell apoptosis induction by scFvC54:sTRAIL is caspase-8 specific. A: Ramos.EGP2 target cells and Ramos bystander cells were mixed at target- to bystander ratio 2:3 and treated with scFvC54:sTRAIL in the presence or absence of caspase-8 inhibitor Z-IETD-FMK. After 16 h, cells were harvested and apoptosis induction was separately evaluated by loss of \( \Delta \Psi \) in Ramos.EGP2 target cells and Ramos bystander cells. B: Ramos bystander cells ectopically over-expressing c-FLIP \(_L\) are largely insensitive to bystander apoptosis induction. Ramos.EGP2 target cells were mixed at target- to bystander ratio 2:3 with either parental Ramos bystander cells or with Ramos bystander cells ectopically overexpressing c-FLIP \(_L\) (Ramos.c-FLIP \(_L\)). Mixed cultures were subsequently treated with 300 ng/ml scFvC54:sTRAIL in the presence or absence of MAb MOC31, after which apoptosis induction was evaluated in bystander Ramos or Ramos.c-FLIP \(_L\) by loss of \( \Delta \Psi \). All values indicated in the graph are mean + standard error of the mean of four independent experiments.

![Fig. 4](image)

**Fig. 5.** Separate evaluation of caspase activation and PARP cleavage in Jurkat.EGP2 target cells and Jurkat bystander cells. Jurkat.EGP2 target cells and Jurkat bystander cells were mixed at target to bystander ratio 1:1 and treated for 6 h with scFvC54:sTRAIL, in the presence or absence of MAb MOC31 or MAb 2E5. After treatment, Jurkat.EGP2 target and Jurkat bystander cells were separated by high-speed cell sorting, after which A: Jurkat.EGP2 target cells and B: Jurkat bystander cells were separately analyzed by immunoblot for caspase-8 activation, caspase-3 activation, and PARP degradation. Arrows indicate bands corresponding to cleaved caspase-8. Of note, in the caspase-8 blot of both Jurkat.EGP2 target cells and Jurkat bystander cells a specific band derived from the heavy chain of MAb MOC31 is also visible.

![Fig. 5](image)
protein PARP (Fig.5A and B, lane 2). Treatment in the presence of MAb MOC31 or MAb 2E5 inhibited caspase activation and PARP cleavage in both target and bystander cells (Fig.5A and B, lane 3 and 4, respectively). Neither caspase activation nor PARP cleavage was observed when Jurkat bystander cells were treated in the absence of Jurkat.EGP2 target cells, even when treatment was prolonged to 24 h (data not shown).

Fluorescent microscopy of bystander apoptosis induction
Microscopic evaluation of untreated mixed cultures revealed that adherent U87MG.EGP2 target cells and U87MG bystander cells were interconnected by cellular protrusions (Fig.6A). Protrusions coming from U87MG.EGP2 target cells can be appreciated due to the EGFP-fluorescence present in the cytoplasm of these cells. When
a mixed culture of U87MG.EGP2 target cells and U87MG bystander cells (ratio 1:4) was treated with 300 ng/ml scFvC54:sTRAIL for 16 h, pronounced apoptotic morphological features such as membrane blebbing and nuclear condensation were visible in both target and bystander cells. The efficacy of the bystander effect was apparent from the fact that apoptotic morphology was observed in almost all U87MG bystander cells (Fig.6B). Identical treatment in the presence of either MAb MOC31 or MAb 2E5 strongly inhibited the appearance of apoptotic morphology in both U87MG.EGP2 target cells and U87MG bystander cells (Fig.6C and D).

**No innocent bystander apoptosis in isolated PBMCs**

Treatment of mixed cultures of isolated leukocytes (innocent bystander cells) and Jurkat.EGP2 cells with scFvC54:sTRAIL did not lead to any significant induction of apoptosis in the bystander leukocytes (Fig.7), whereas apoptosis of up to 46% was observed in Jurkat.EGP2 target cells. Apoptosis in Jurkat.EGP2 was specifically inhibited when treatment was performed in the presence of MAb MOC31 or MAb 2E5.

**Discussion**

It has been shown that crosslinking of TRAIL-receptors is crucial for the efficient induction of apoptosis in tumour cells. Previously, we reported on target cell-restricted fratricide apoptosis induction by the fusion protein scFvC54:sTRAIL, due to the efficient
crosslinking of agonistic TRAIL receptors TRAIL-R1 and TRAIL-R2. In the present study we analyzed whether selective binding of scFvC54:sTRAIL to EGP2-positive tumour cells could further be used to crosslink TRAIL-receptors on neighbouring tumour cells devoid of EGP2 expression using mixed cell culture experiments. To this end, we selected a series of cell lines that represent three major human malignancies: acute lymphoblastic T cell leukemia (Jurkat), B cell lymphoma (Ramos), and Glioblastoma Multiforme (U87MG), all of which are EGP2-negative and generated EGP2-positive target cells thereof by retroviral transduction. Furthermore, we devised a method that allowed for distinctive evaluation of apoptosis in target and bystander cells.

All EGP2-negative bystander cell types used were fully resistant to prolonged treatment with scFvC54:sTRAIL (16h, 300 ng/ml). However, when mixed cultures of EGP2-positive target cells and corresponding EGP2-negative bystander cells were treated, potent pro-apoptotic effects of up to 80% apoptosis induction were achieved in EGP2-negative bystander cells (Fig.3A). Pro-apoptotic bystander activity of scFvC54:sTRAIL was observed for both suspension tumour cell types (Jurkat and Ramos) and for adherent U87MG glioblastoma cells (Fig.3C).

Treatment of mixed cultures containing as little as 1% of EGP2-positive target cells still showed significant apoptosis induction of up to 17% in EGP2-negative bystander cells. This clearly indicated that significant pro-apoptotic bystander activity of scFvC54:sTRAIL can be achieved at low target- to bystander cell ratios. Treatment in the presence of an EGP2-blocking antibody or a TRAIL-neutralizing antibody strongly inhibited apoptosis induction in both target and bystander cells at all ratios analyzed (Fig.3A and B). When treatment was performed using identical amounts of a scFv:sTRAIL fusion protein of irrelevant specificity (Mock-scFvH22:sTRAIL), no induction of apoptosis was observed (Fig.3C). Furthermore, apoptosis induction was specifically absent in bystander cells that ectopically over-expressed c-FLIPL, a specific inhibitor of death receptor-induced apoptosis (Fig.4B). Immunoblot analysis of post treatment-sorted target and bystander cells demonstrated identical activation profiles of caspase-3 and caspase-8, and cleavage of PARP (Fig.5A and B). Together these results all indicated that both fratricide and bystander apoptosis induction by scFvC54:sTRAIL is mediated by target cell-dependent inter-cellular crosslinking of agonistic TRAIL-receptors.

Microscopic evaluation of a mixed culture (ratio 1:4) of adherent U87MG.EGP2 target cells and U87MG bystander cells treated with scFvC54:sTRAIL visualized pronounced apoptotic morphological features (nuclear condensation and membrane blebbing) in both target and bystander cells. The strong bystander effect observed here might partly be due to the
fact that U87MG cells have extensive cellular protrusions that appear to make multiple intracellular connections even to more distant cells (Fig.6A). Possibly, this particular cell morphology influences TRAIL-receptor crosslinking by scFvC54:sTRAIL between interconnected target- and bystander cells. It is tentative to speculate that scFv:sTRAIL treatment of target cells with more extensive cellular protrusions may induce apoptosis in more distant bystander cells.

As discussed above, we analyzed the pro-apoptotic bystander effect by scFvC54:sTRAIL down to extremely low target to bystander cell ratios. We noticed that when treatment was performed at ratios < 1:10, apoptosis induction in the target cells was partly diminished (Fig.3A). It appears that the presence of a vast majority of bystander cells reduces direct cellular contacts between EGP2-positive target cells, subsequently reducing fratricide apoptosis induction of these cells. The inhibitory effect of bystander cells on fratricide apoptosis induction in target cells was not observed at higher and possibly more realistic target-to-bystander cell ratios.

Previously, bystander effects have been observed in ADEPT (antibody-directed enzyme prodrug therapy) and VDEPT (virus-directed enzyme prodrug therapy), therapeutic approaches that target a non-human prodrug-converting enzyme into tumour cells and involve the transfer and diffusion of toxic metabolites from one cell to another. Usually, the toxic metabolites produced using these strategies cannot freely transit the cell membrane. Consequently, these bystander effects chiefly depend on GJIC between target and bystander tumour cells. Unfortunately, most cancer cells lack functional GJIC. The bystander apoptosis activity described here for scFvC54:sTRAIL does not require internalization, enzymatic conversion, diffusion, or gap junctional intercellular communication (GJIC) between target and bystander cells.

An additional problem in both ADEPT and VDEPT appears to be the preferential killing of targeted cells due to their relative high intracellular concentration of the toxic metabolite, resulting in a decreased bystander effect. In contrast, the bystander activity of scFvC54:sTRAIL is likely to be maintained during the whole process of target cell apoptosis induction. Moreover, apoptosis of a given target cell can yield numerous minute apoptotic bodies with intact EGP2-positive cellular membranes. In vitro, target cell-derived apoptotic bodies displaying scFvC54:sTRAIL might continue to contribute to the crosslinking of TRAIL receptors and potentially disseminate the bystander effect to more distant tumour cells. The presence and subsequent contribution of such apoptotic bodies to the bystander effect studied here remains to be clarified. However, in vivo it is likely that phagocytosing cells of the immune system rapidly scavenge such apoptotic bodies.
before additional bystander apoptosis induction is initiated.

We wondered whether the potent pro-apoptotic bystander effect of scFvC54:sTRAIL might also result in the killing of ‘innocent bystander’ cells such as normal blood cells. Therefore, we added freshly isolated leukocytes to various bystander experiments and found no significant signs of apoptosis induction in the various blood cell types (Fig.7). This indicates that at least in this experimental setting scFvC54:sTRAIL has retained its tumour selective apoptosis activity with no signs of innocent bystander apoptosis induction. Nevertheless, from the present study it cannot be excluded that scFvC54:sTRAIL might exert toxic or innocent bystander effects towards other normal cells and tissues. Toxicity studies of scFvC54:sTRAIL can possibly be performed in our human EGP2 transgenic mouse model\(^6\) in which human EGP2 expression displays authentic expression patterns in mouse epithelia.

In conclusion, this is the first example of target cell-dependent bystander apoptotic activity by a scFv:sTRAIL fusion protein. Further development of this new principle is warranted for TRAIL and antibody-based therapy of human cancers that escape current antibody-based therapy due to heterogeneous target antigen expression.

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References


