CD70-restricted specific activation of TRAILR1 or TRAILR2 using scFv-targeted TRAIL mutants

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To combine the CD27 stimulation inhibitory effect of blocking CD70 antibodies with an antibody-dependent cellular cytotoxicity (ADCC)-independent, cell death-inducing activity for targeting of CD70-expressing tumors, we evaluated here fusion proteins of the apoptosis-inducing TNF family member TRAIL and a single-chain variable fragment (scFv) derived from a high-affinity llama-derived anti-human CD70 antibody (lzxC70). A fusion protein of scFv:lzxC70 with TNC-TRAIL, a stabilized form of TRAIL, showed strongly enhanced apoptosis induction upon CD70 binding and furthermore efficiently interfered with CD70-CD27 interaction. Noteworthy, introduction of recently identified mutations that discriminate between TRAILR1 and TRAILR2 binding into the TRAIL part of scFv:lzxC70-TNC-TRAIL resulted in TRAIL death receptor-specific fusion proteins with CD70-restricted activity.

Cell Death and Disease (2014) 5, e1035; doi:10.1038/cddis.2013.555; published online 30 January 2014

Subject Category: Cancer

CD70 (CD27L) is a typical member of the tumor necrosis factor (TNF) family and thus occurs primarily as a trimeric type II transmembrane protein. CD70 expression is normally restricted to B cells, mature dendritic cells and activated T cells.1,2 CD70 interacts with CD27, a member of the TNF receptor superfamily that is expressed on a subset of B-cells, NK and NKT cells but especially on various types of T cells.1,2 Although there is some evidence that CD70 itself directly triggers intracellular signaling in CD70+ cells in response to CD27 binding (reverse signaling), the huge majority of CD70-mediated effects are based on activation of CD27-associated signaling pathways. Unusual for a member of the TNF receptor family, CD70 is expressed as a disulfide-bonded homodimer instead as a monomer, although there is evidence that at least some of the ‘normal’ monomeric TNF receptor types also undergo auto-aggregation with the help of a pre-ligand assembly domain (PLAD) in their extracellular domain.3,4 CD27 stimulation results in recruitment of Siva-1, a poorly understood apoptosis regulatory molecule, and of the adapter protein E3 ligases TRAF2 and TRAF5.5–7 In accordance with the established role of TRAF2 and TRAF5 in NFκB signaling, CD27 activation results in enhanced activity of the classical and alternative NFκB pathway, which both has been implicated in survival, activation and differentiation of T cells.9,10

CD70 is also strongly expressed with high frequency in a variety of hematologic malignancies and surprisingly often in solid tumors, especially in clear cell renal cell carcinoma but also frequently (10–25%) in pancreatic, larynx, ovarian and colon cancer, glioblastoma and melanoma.11–14 The reasons and functional relevance of CD70 expression in transformed cells are yet poorly understood and may differ from case to case. At the first glance, the expression of CD70 on tumor cells appears counterintuitive as this should result in enhanced antigenicity of the tumor. Indeed, it has been demonstrated in animal models that treatment with agonistic CD27 antibodies or vaccinia virus-delivered CD70 enhances anti-tumor T-cell responses.15–18 Furthermore, it has been demonstrated that CD70-secreting tumor cells induce an enhanced anti-tumor immune response.19,20 However, especially in situations of persistent CD70 expression, immune inhibitory effects may also appear, because of exhaustion of the T-cell pool and there is further evidence that tumor cells expressing CD70 increase the amount of Tregs in the tumor microenvironment.21,22 Irrespective from its concrete function in the tumor cell, due to its quite restricted expression on non-transformed tissue, CD70 can be considered as an excellent target for therapeutic antibodies. It is therefore no surprise that CD70-specific antibodies are under clinical and preclinical investigation for the treatment of autoimmune diseases and cancer23 (http://clinicaltrials.gov/).

With respect to CD70 targeting in cancer, two conceptions are of particular relevance: first, the very well-established idea to exploit CD70 as a tumor marker to direct ADCC-inducing antibodies or antibody–drug conjugates to the malignant cells, which is already in clinical trials, and, second, the relatively new strategy to block the putative immune inhibitory effects of tumor cell-expressed CD70. Although the latter aim could be similarly achieved by ADCC-mediated tumor cell destruction, CD70-blocking antibodies may elicit these effects also at lower concentrations insufficient to compensate for the
inhibitory effect of the endogenously present serum IgG in the presence of ADCC inhibitory signals/molecules.

TNF-related apoptosis-inducing ligand (TRAIL) is a member of the TNF ligand family with potent apoptosis-inducing properties and attracts considerable interest due to its potential use for tumor therapy.24,25 This is because of the finding that most nontransformed cells are for various reasons protected from TRAIL-induced apoptosis, whereas many transformed cells are TRAIL sensitive. Similar to other TNF ligands, TRAIL is initially expressed as a membrane-bound trimeric ligand that signals apoptosis by activation of the death receptors TRAILR1 and TRAILR2. The soluble ectodomain of TRAIL also assembles into trimeric molecules but is in contrast to the membrane-bound molecule poorly active despite receptor binding.26,27 It has been shown that the poor responsiveness of TRAIL death receptors (particular of TRAILR2) toward soluble TRAIL trimers can be overcome in two different ways. By oligomerization of two or more TRAIL trimers or second by artificial cell surface immobilization, for example, by fusing soluble TRAIL to a single-chain variable fragment (scFv) of an antibody specific for a cell surface-exposed antigen.28 Noteworthy, the latter principle not only stimulates receptor activity but also increases the concentration of soluble TRAIL toward soluble TRAIL trimers can be overcome in vivo.29–31 Most importantly, the result of these findings is that the agonistic activity of FcR1b strongly enhances the agonistic activity of TRAILR1/2-specific antibodies.30–35 Most important, in vivo studies have demonstrated that Dulanermin unleashes the full agonistic activity of TRAIL death receptors TRAILR1 and TRAILR2 discriminating TRAIL variants. These bifunctional proteins not only overcome the poor activity of soluble TRAIL in a CD70-restricted manner but also interfere with CD70–CD27 interaction, which in CD70−/−cancers may have protumoral activities.

**Results**

**CD70 cell surface expression.** CD70 expression in the healthy organism is strongly restricted to T cells, some types of B cells and NK cells. Reports showing strong expression of CD70 in B-cell lymphomas and various types of solid tumors defined this molecule as a potential target for tumor therapy.11–14 Most data on CD70 expression are based on immunohistochemistry techniques that do not discriminate between cell surface and intracellularly expressed CD70. We thus screened initially a large panel of tumor cell lines with respect to cell surface expression of CD70 by FACS (Table 1). B-cell lymphoma cell lines were with high frequency (>90%) strongly positive for cell surface-expressed CD70, whereas only a subfraction of glioblastoma, pancreatic, ovarian, renal and melanoma cell lines was positive for cell surface CD70.

**Table 1** Cell surface expression of CD27 and CD70 in cancer cell lines

| Cell line          | Type     | CD70      | CD27      |
|--------------------|----------|-----------|-----------|
| Raji               | NHL      | +         | + +       |
| U266               | MM       | +         |           |
| Amo-1              | MM       | + + +     |           |
| RPMI               | MM       | +         | - / +     |
| IN-2                | MM       |           |           |
| KMS-11             | MM       |           |           |
| MM1.2              | MM       |           |           |
| KMS12.BM           | MM       | +         | + + +     |
| JNN3               | MM       |           |           |
| Mino               | MCL      |           |           |
| Jeko-1             | MCL      |           |           |
| Rec-1              | MCL      |           |           |
| Granta-519         | MCL      |           |           |
| MJ(G11)            | CTCL     |           |           |
| JVM3               | CLL      |           |           |
| Ramos-Ra1          | Burkitt  |           |           |
| BJAB               | Burkitt  | +         | + + +     |
| Daudi              | Burkitt  | +         | - / +     |
| SuDHL6             | DLBCL    |           |           |
| Panc-1             |          |           |           |
| Panc89             |          |           |           |
| PancTu1            |          |           |           |
| MiaPaca-2          |          |           |           |
| Colo357            |          |           |           |
| A498               |          | +         | + +       |
| 786-O              |          | +         | + +       |
| FaDu               | SCC      |           |           |
| HL-A-76            | SCC      |           |           |
| WM-35              |          |           | - / +     |
| WM-3211            |          |           |           |
| WM-115             |          | +         | + + +     |
| WM-1552C           |          | +         | + + +     |
| WM793              | Melanoma | - / +     |           |
| WM-3248            |          | +         |           |
| SK-mel-19          | Melanoma |           |           |
| MV3                | Melanoma |           |           |
| WM-852             | Melanoma |           |           |
| WM-451-LU          | Melanoma |           |           |
| WM-1205Lu          | Melanoma |           |           |
| BLM                | Melanoma |           |           |
| OAW-42             | Ovarian cancer | - / + |           |
| SKOV-3             | Ovarian cancer | - / + |           |
| OVCAR3             | Ovarian cancer | - / + |           |
| GaMG               | Globlastoma | - / + |           |
| U87                | Globlastoma | +         |           |
| U343               | Globlastoma | +         |           |
| Ln18               | Globlastoma | +         |           |

Abbreviations: CLL, chronic lymphocytic leukemia; CTCL, cutaneous T-cell lymphoma; DLBCL, diffuse large B-cell lymphoma; MCL, mantle cell lymphoma; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; RCC, renal cell carcinoma; SCC, squamous cell cancer. Cells were analyzed by using FACS. + + + indicates very high expression, for example, like Mino cells (Figure 5A). + + indicates high expression, for example, like OVCAR3 cells (Figure 5A). + indicates significant expression, − indicates lack of significant binding.
Initial characterization of a novel llama-derived human CD70-specific antibody. By screening for binding to recombinant human CD70, we isolated the human CD70-specific antibody 27B3, designated in the following as LzCD70, from an antibody phage display library constructed from peripheral blood cells of llamas immunized with the human renal cell carcinoma cell line 786-O. LzCD70 blocks CD70–CD27 interaction with high efficiency and in this respect is at least as potent as 1F6 (Figure 1a), another CD70-specific antibody for which sequence information is available from public patent databases (US 2006/0083736A1) and for which a drug-conjugated variant (SGN-75) is currently under clinical investigation (http://clinicaltrials.gov). We further confirmed the inhibitory effect of LzCD70 on CD70–CD27 interaction in a functional assay where CD70-expressing cells (U266 and Mino) have been used to stimulate CD27 on HT1080 cells stably transfected with this receptor. Activation of CD27 in the HT1080-CD27 cells results in an increase in IL8 production. As expected, LzCD70 and 1F6 inhibited the increase in IL8 production by HT1080-CD27 cells, which was induced by coculturing with CD70-expressing U266 or Mino cells (Figure 1b).

For binding studies, we generated a monomeric and a trimeric fusion protein of the scFv variant of LzCD70 with the Gaussia princeps luciferase (GpL). Trimerization of scFv:LzCD70 was achieved by fusing the C terminus of the scFv to the trimerization domain of tenascin-C (TNC). The GpL domain was furthermore introduced to facilitate binding studies with the scFv:LzCD70 variants. Cellular equilibrium binding studies with the monomeric scFv:LzCD70 protein and the trimeric scFv:LzCD70-TNC-GpL molecule on Mino and OVCAR3 cells yield $K_D$ values of 2300 and 1300 pM for the monomeric scFv variant and of 160 and 240 pM for the trimeric variant (Figure 1c).

In sum, these data not only confirmed the high affinity of LzCD70 for cells expressing CD70 but also demonstrated the principle functionality of the corresponding scFv upon TNC-driven trimerization. The latter was important as it was intended in the following to use a TNC-stabilized variant of TRAIL for the construction of CD70-targeted TRAIL fusion proteins.

**Characterization of TRAILR1- and TRAILR2-specific TRAIL mutants by cellular binding studies.** It was envisaged in this study to equip a blocking CD70-specific

![Figure 1](image-url)
scFv with TRAIL as well as TRAILR1- and TRAILR2-specific TRAIL mutants for three reasons. First aim was to overcome the limited activity of soluble TRAIL by anchoring to CD70. The second intention was to link this with inhibition of CD70-mediated protumoral activities. Third, against the background of the safe and also limited activity of the TRAIL death receptor-targeting reagents currently under clinical investigation (see introduction), it appears possible that cell surface-anchoring TRAIL variants with high-specific activity cause unanticipated side effects on nontransformed cells not recognized so far. In this speculative case, the use of TRAIL death receptor-specific TRAIL mutants could serve as an option to reduce potential side effects related to activation of the TRAIL death receptor type not relevant for apoptosis induction in the targeted tumor type. Indeed, several types of tumors are particularly sensitive for apoptosis induction via one of the two TRAIL death receptors; for example, chronic lymphocytic leukemia (CLL) and pancreatic tumors are particularly TRAILR1 sensitive. Moreover, there is initial evidence from a recent study that TRAIL death receptor-specific mutants have higher specific activity than the wild-type molecule. Therefore, we introduced recently published mutations in our basic TNC-TRAIL construct conferring specificity for TRAILR1 (G131R/R149/S159R/N199R/K201H/S215D) and TRAILR2 (Y189Q/R191K/Q193R/H264R/I266L/D267Q). The receptor specificity of these TRAIL mutants has so far mainly been analyzed by cell-free approaches. We therefore analyzed the TRAIL receptor usage of TRAILR1 and TRAILR2 in cellular equilibrium binding studies. For this purpose, we tagged the TNC-TRAIL variants with an N-terminal luciferase (GpL) domain and performed equilibrium binding studies with HEK293 cells transiently transfected with TRAILR1, TRAILR2, TRAILR3 and TRAILR4. Mock-transfected HEK293 cells, which express no TRAILR1 and TRAILR3 and which have only moderate expression of TRAILR2 and TRAILR4 (Supplementary Figure 1), were analyzed in parallel to determine nonspecific binding. For GpL-TNC-TRAIL, we obtained $K_D$ values for TRAILR1 and TRAILR2 of 3450 and 880 pM. The $K_D$ value of interaction of GpL-TNC-TRAILmutR1 with TRAILR1 was 2590 pM (Figure 2a). There was also significant binding to TRAILR2-transfected cells with GpL-TNC-TRAILmutR1, but the maximal specific binding was significantly lower than with GpL-TNC-TRAILmutR2 and GpL-TNC-TRAIL (Figure 2a). This suggests that the binding is not primarily caused by binding to TRAILR2 but rather reflects...
binding to endogenous TRAILR4 and/or heterocomplexes of TRAILR2 and TRAILR4. Indeed, GpL-TNC-TRAILmutR1, in contrast to GpL-TNC-TRAIL and GpL-TNC-TRAILmutR2, failed to bind to Jurkat cells, which only express TRAILR2 (Figure 2b). The affinity of the interaction of GpL-TNC-TRAILmutR2 with TRAILR2 was 720 pM, whereas there was no detectable binding to TRAILR1 (Figure 2a). GpL-TNC-TRAILmutR1 also interacted with TRAILR3 and TRAILR4, whereas GpL-TNC-TRAILmutR2 failed to bind TRAILR3 (Figure 2a). The efficient discrimination of GpL-TNC-TRAILmutR1 and GpL-TNC-TRAILmutR2 between the two TRAIL death receptors was also evident from in vitro binding studies with immobilized TRAILR1-Fc and TRAILR2-Fc (Figure 2c). To further prove that the high preference of GpL-TNC-TRAILmutR1 and GpL-TNC-TRAILmutR2 for TRAILR1 and TRAILR2 indeed translates into discriminated death receptor signaling, we performed immunoprecipitation experiments. For these purposes, we used Fc-fusion proteins of TRAIL, TRAILmutR1 and TRAILmutR2. The fusion of the various TRAIL variants with the human IgG1 Fc domain resulted in the formation of hexameric proteins and not only allowed easy immune precipitation of ligand-bound receptor complexes but also substituted for the known need of oligomerization of soluble trimeric TRAIL variants to achieve optimal activity. In accordance with the results from the binding studies, there was practically no TRAILR2 in Fc-TRAILmutR1 immunoprecipitates and no detectable levels of TRAILR1 in Fc-TRAILmutR2 immunoprecipitates, whereas both receptors were easily detectable in immunoprecipitates of Fc-TRAIL-stimulated cells (Figure 2d). We also analyzed cell death induction using the TNC-TRAIL variants with and without anti-Flag mAb M2 oligomerization in the Jurkat T-cell line, which as mentioned above only expresses TRAILR2, and with Mino and OVCAR3 cells that both express TRAILR1 and TRAILR2 (Figure 3a). Viability assays as well as western blot analysis of caspase processing confirmed the strong TRAILR1/TRAILR2 preference of the TRAILmutR1 and TRAILmutR2 variants (Figures 3b and c). As observed in former studies, anti-Flag oligomerization of the Flag-tagged TNC-TRAIL variants resulted in a strong increase in cell death induction (Figure 3b).

**scFv:**shCD70-TRAIL variants display CD70-restricted activation of TRAILR1 and TRAILR2. We and others previously found that cell surface antigen-specific scFv-TRAIL fusion proteins not only allow targeting of TRAIL activity to tumor antigen-expressing cells but also that the TRAIL domain exerts a higher apoptosis-inducing activity to tumor antigen-expressing cells but also that the TRAIL fusion proteins not only allow targeting of TRAIL previously found that cell surface antigen-specific scFv-TRAIL domain exerts a higher apoptosis-inducing activity to tumor antigen-expressing cells but also that the TRAIL fusion proteins not only allow targeting of TRAIL induced significant cell death in CD70 and mock-transfected HT1080 cells. More important, the ED50-value of scFv:**shCD70-TRAIL and scFv:**shCD70-TRAILmutR2 for apoptosis induction in the CD70-transfected cells almost increased one order of magnitude when binding to CD70 was blocked with a conventional CD70-specific antibody, whereas CD70 blockade showed no effect on the dose response relation of apoptosis induction in the mock-transfected HT1080 cells (Figure 4c). This indicated that binding to CD70 resulted in enhanced activation of TRAILR2. Next, we analyzed the scFv-TRAIL fusion proteins using CD70 Jurkat cells and Mino and OVCAR3 cells showing endogenous CD70 expression (Figure 5b). To uncover a potential CD70-restricted apoptotic activity, cells were treated in the absence and presence of conventional CD70-specific antibodies competing with scFv:**shCD70 for CD70 binding. In contrast to the TNC-TRAIL and TRAILmutR2 variants (Figure 3b), the corresponding scFv:**shCD70-TRAIL fusion proteins showed some cell death induction in Jurkat cells at higher concentrations, but this was not affected by the cotreatment with the blocking CD70 antibody (Figure 5c). Therefore, the moderate CD70 binding-independent cytotoxicity of scFv:**shCD70-TNC-TRAIL and scFv:**shCD70-TNC-TRAILmutR2 on Jurkat cells most likely reflects the activity of a minor subspecies of aggregated fusion proteins mimicking anti-Flag-activated TNC-TRAIL. More important, in the CD70-expressing Mino and OVCAR3 cells, the CD70-targeted scFv-TRAIL fusion proteins triggered complete cell death at low concentrations (1–10 ng/ml), and the dose response of cell death induction shifted to 10- to 100-fold higher concentrations when access to CD70 was blocked by competing a CD70 antibody (Figure 5b). In agreement with enhanced TRAIL death receptor activation upon CD70 binding, caspase-8 activation and processing of the caspase-8 substrates BID and caspase-3 by the various scFv-TRAIL fusion proteins were significantly diminished by blockade of CD70 (Figure 7c). Similarly, there was strongly reduced processing of the effector caspase substrate PARP1 indicative for reduced apoptosis (Figure 5c). We also evaluated the apoptotic activity of mixtures of trimeric TNC-TRAIL with high concentrations of scFv:**shCD70-TNC-GpL, the trimeric variant of the scFv:**shCD70 antibody fragment already described in Figure 1c. From these experiments, no evidence could be found that CD70 blockade by scFv:**shCD70-TNC-GpL interferes with cell death induction by TNC-TRAIL or oligomeric TNC-TRAIL (Figure 6a). However, as expected, scFv:**shCD70-TNC-GpL inhibited the apoptotic activity of our scFv-CD70-TRAIL fusion proteins on CD70 cells. Therefore, when the CD70-targeting scFv domain and the TRAIL domain are separated into two distinct molecules
without change in the trimeric organization of the domains as present in the TRAIL fusion proteins, there is no CD70-dependent increase in the apoptotic activity of TRAIL. As scFv:lsCD70-TNC-GpL is equally effective as our scFv:lsCD70-TRAIL fusion proteins in CD70 binding, this rules out that blocking of CD27–CD70 interaction or triggering of reverse CD70 signaling contribute to cell death induction by the scFv:lsCD70-TNC-TRAIL fusion proteins. We also evaluated cell death induction by scFv:lsCD70-TRAIL in Raji and KMS12BM cells, which coexpress CD70 and CD27 (Table 1, Figure 6b). There was again a strong reduction in cell death induction when CD70 was blocked with a competing CD70-specific antibody (Figure 6c), but the effect of CD70 binding appears less strong in these cell lines compared with the CD27-negative cell lines Mino and OVCAR3. It is tempting to speculate that this is related to the fact that the fusion protein has to compete here with endogenous CD27 for CD70 binding but other factors (e.g. different expression levels) cannot be fully ruled out.

**Discussion**

Targeting of CD70 and TRAIL death receptors to treat cancer is currently evaluated in various preclinical studies and phase I clinical trials. In case of CD70 targeting, it was the initial idea to exploit the tumor-associated expression of CD70 to direct antibody-dependent effector functions to tumor cells. The recent recognition of the immune suppressive activities of cancer cell expressing CD70, however, now also caused an interest in CD70 blockade as a therapeutic option in cancer therapy. In case of targeting of the two TRAIL death receptors, the therapeutic rational was initially apoptosis induction in tumor cells, but now there is evidence that the tumor vasculature is similarly a useful TRAIL target structure.

**Figure 3** Apoptosis induction by TRAILR1 and TRAILR2 discriminating TRAIL mutants. (a) OVCAR3, Mino and Jurkat cells were analyzed with respect to cell surface expression of TRAILR1 and TRAILR2 by FACS. (b) OVCAR3, Mino and Jurkat cells were challenged in triplicates with the indicated concentrations of Flag-TNC-TRAIL, Flag-TNC-TRAILmutR1 and Flag-TNC-TRAILmutR2 in the presence and absence of 1 μg/ml of the anti-Flag mAb M2. Next day, cellular viability was determined using the MTT assay or crystal violet staining. OVCAR3 cells were challenged in the presence of 2.5 μg/ml CHX, which sensitizes this cell line for apoptosis induction. (c) The indicated cell lines were treated for 4–6 h with 100 ng/ml of the various Flag-TNC-TRAIL variants oligomerized with 1 μg/ml of the anti-Flag mAb M2, and total cell lysates were assayed by western blotting for the presence of the indicated proteins. OVCAR3 cells were again sensitized for apoptosis induction by CHX treatment.
Immunostimulation and cell death induction in tumor, or tumor-associated, cells are two complementary and maybe even synergistic aims in tumor therapy. Combination therapies addressing both of these aims are therefore particularly attractive. To obtain a single molecule that concomitantly allows blockade of the immunosuppressive activities of tumor cell-expressing CD70 and stimulation of cell death in tumors, we genetically fused TRAIL with the CD70-blocking scFv: hCD70 (Figure 1). Moreover, in view of former studies, showing superior activity of cell surface antigen-bound TRAIL fusion proteins compared with conventional soluble TRAIL, we anticipated enhanced cell death induction by the fusion protein when bound to CD70. In fact, the resulting fusion protein, scFv: hCD70-TRAIL, showed the targeted combinational activities and not only blocked CD27-CD70 interaction but also triggered cell death 10- to 100-fold more efficiently in the presence of cells expressing CD70 (Figure 5). Superior receptor activation by interaction with cell surface antigen-bound ligand molecules or naturally occurring membrane-bound ligand molecules has also been observed for various other members of the TNF receptor family but does not represent a common feature of this receptor family. To clarify whether both or only one of the two TRAIL death receptors possess improved responsiveness to CD70-bound scFv-targeted receptor-specific TRAIL mutants, we introduced published mutations in the TRAIL part of the molecule that confer preference for TRAILR1 or TRAILR2 (Figures 2 and 3). Enhanced cell death induction upon CD70 binding was found with the TRAILR1- as well as with the TRAILR2-preference variant of scFv: hCD70-TRAIL (Figure 5). This suggests that the superior activity of CD70-bound (or generally cell surface antigen-bound) TRAIL fusion proteins can be exploited, irrespective of having tumors that are only sensitive for one of the two TRAIL death receptors.
Figure 5  CD70-restricted apoptosis induction by scFv:shCD70-TRAIL, scFv:shCD70-TRAILmutR1 and scFv:shCD70-TRAILmutR2. (a) The indicated cell lines were analyzed by using FACS for CD70 cell surface expression. (b) OVCAR3, Mino and Jurkat cells were cultured in 96-well plates, and half of the cells were pretreated with 10 ng/ml of a scFv:shCD70 competing conventional CD70-specific antibody. Cells were then challenged overnight in triplicates with the indicated concentrations of scFv:shCD70-TNC-TRAIL, scFv:shCD70-TNC-TRAILmutR1 and scFv:shCD70-TNC-TRAILmutR2. Finally, cellular viability was determined using the MTT assay or crystal violet staining. OVCAR3 cells were challenged in the presence of 2.5 µg/ml CHX, which sensitizes this cell line for apoptosis induction. (c) The indicated cell line cells were stimulated in the presence and absence of the conventional CD70-specific antibody shCD70 for 4–6 h with 100 ng/ml of scFv:shCD70-TNC-TRAIL, scFv:shCD70-TNC-TRAILmutR1 and scFv:shCD70-TNC-TRAILmutR2. Total cell lysates were analyzed by western blotting for processing of the indicated proteins.
Soluble TRAIL (Dulanermin) and various TRAIL death receptor-specific IgG1 antibodies are currently under clinical investigation and were found so far to be well tolerated even if applied in combination with other drugs. In view of the limited activity of soluble TRAIL and the need of anti-TRAIL death receptor antibodies to bind to FcγRIIb to become strongly agonistic in vivo, this good tolerability is in retrospect perhaps no surprise. Thus, more potent TRAIL death receptor-targeting reagents, such as oligomerized TRAIL, TRAIL fusion proteins with the capability to anchor to the cell surface or engineered TRAIL death receptor antibodies with high affinity for FcγRIIb may have the potential to elicit improved anti-tumor activity, but this may come along with a risk for so far not recognized side effects. In this context,
the fusion proteins presented in our study not only solve the problem of poor specific activity of soluble TRAIL trimers by cell surface anchoring but also reduce the danger of potential side effects by using the tumor-associated target CD70 for this purposes. The use of a CD70-blocking protein domain for cell surface TRAIL, anchoring furthermore links TRAIL death receptor stimulation with a possibly breach of the tumor-associated immune homeostasis. Indeed, recent findings showed that CD27 activation reduces apoptosis of regulatory T cells in the thymic medulla and also in CD70-expressing solid tumors, and in the latter case this led to enhanced tumor growth. Therefore, cancer types where the presence of Tregs is of poor prognosis could be preferential targets for CD70-targeted TRAIL fusion proteins. CD70-targeted TRAIL constructs may also affect immune cells out of the tumor area but to which extent this compromises potential anti-tumoral activities of the CD70-targeting TRAIL fusion proteins can ultimately only be verified in future animal studies with a humanized immune system or using mouse CD70-targeted TRAIL fusion proteins.

Material and Methods

Cell lines and reagents. The INA6 cell line was a gift from Martin Gramatzki (Kiel, Germany) and has been described elsewhere. All other cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) or ATCC. Cells were cultivated in RPMI1640 medium (PA, Colbe, Germany) supplemented with 10% heat-inactivated fetal bovine serum (PAA). For cultivation of INA6 cells, recombinant human IL6 (Immunotools, Friesoythe, Germany) was added to the medium to reach a concentration of 10 ng/ml.

Molecular cloning, production and purification of recombinant proteins. A pCR2-based (Invitrogen) expression plasmid encoding for Flag-TNC-TRAIL with an Ig leader sequence has been published elsewhere. To introduce published mutations that allow discrimination between TRAILR1 and TRAILR2 binding, the TRAIL part in pCR3-Flag-TNC-TRAIL was replaced by synthetic genes encoding corresponding TRAIL fragments harboring the mutations of interest. The TRAIL mutant Y189Q/R191K/Q193R/H264R/I266L/D267Q for which strongly reduced binding to TRAILR2 has been published was termed TRAILmutR1. To obtain the GpL-TNC-TRAILmutR1 and GpL-TNC-TRAILmutR2 for 1 h at 37°C. Unbound molecules were removed by five washing cycles with PBS, and the well-associated luciferase activities were finally assayed by help of the Gaussia luciferase Assay Kit. Non-specific binding data were derived from wells incubated with supernatants of mock-transfected cells instead of TRAILR1/2-Fc. Kd values were again obtained by fitting the binding data to a one side specific binding plot using GraphPad Prism 5.

Heterologous competition assays with CD27ed-Fc-GpL. Cells (3–10 × 10^5 cells) were incubated with mixtures of 200 ng/ml CD27ed-Fc-GpL and increasing concentrations of anti-CD70 antibodies for 1 h at 37°C. After washing the cells five times with ice-cold PBS, cell-bound molecules were again quantified by assaying GpL activity with the Gaussia luciferase Assay Kit and a Lucifer 2 Luminoimeter.

FACS analysis. Cell surface expression of CD70, TRAILR1 and TRAILR2 was determined by help of CD27 (clone 57703, BD), CD70 (clone K24-24, BD), TRAILR1 (clone 16803, R&D Systems, Wiesbaden-Nordenstadt, Germany), TRAILR2 (clone 22235, R&D Systems), TRAILR3 (FAB6302P, R&D Systems) and TRAILR4 (FAB633P, R&D Systems) specific antibodies along with corresponding isotype-matched control antibodies using a FACS Calibur (BD Biosciences, Heidelberg, Germany).

Cell death assay. Cells were seeded in 96-well plates (10 × 10^3/well for adherent cell lines and 50 × 10^3/well for suspension cell lines) and were treated the next day as described in the figure legends. The cellular viability of suspended cells was determined with the help of the MTT assay and viability of adherent cells was quantified by crystal violet staining. Measured data of cellular viability were finally normalized by help of viability values of untreated control cells (100%) and cells that were challenged with a toxic mixture of CD95L, TRAIL and cycloheximide (0%).

IL8 Elisa. Cells supernatants were cleared by centrifugation and assayed for their IL8 content by help of a commercially available ELISA kit (BD Biosciences) according to the protocol of the manufacturer.
Western blot analysis and silver staining. Cells were collected, washed once in PBS and resuspended in Laemmli sample buffer. After vortexing and heat denaturation at 90 °C for 5 min, samples were briefly centrifuged to pellet insoluble cell debris and subjected to fractionation by SDS-PAGE. Proteins were transferred to nitrocellulose membranes by semi-dry blotting, and the proteins of interest were detected by help of appropriate primary antibodies, horseradish peroxidase-conjugated secondary antibodies and the Pierce enhanced chemiluminescence (ECL) western blotting detection system (Thermo Fisher Scientific, Bonn, Germany). Protein samples dedicated for silver staining were again fractionated by SDS-PAGE, and subsequently the gels were processed with the Pierce Silver Stain Kit (Thermo Fisher Scientific) according to the protocol of the supplier.

Conflict of Interest
KS is an employee of arGen-X BVBA and HW is a consultant of arGen-X BVBA. KS and HW are co-inventors of CD70-specific antibodies. The remaining authors authors declare no conflict of interest.

Acknowledgements
This work was supported by the Deutsche José Carreras Leukämie-Stiftung e.V. (R/06/17) and the Deutsche Forschungsgemeinschaft (grant WA 1025/24-1). Mohamed El-Mesery is a German Egyptian Research Long Term Scholarship (GERLS) holder funded by DAAD.
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Supplementary Information accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis)