

# 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 (I<sub>xh</sub>CD70). A fusion protein of scFv:I<sub>xh</sub>CD70 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:I<sub>xh</sub>CD70-TNC-TRAIL resulted in TRAIL death receptor-specific fusion proteins with CD70-restricted activity.

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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.<sup>1,2</sup> 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.<sup>1,2</sup> Although there is some evidence that CD70 itself directly triggers intracellular signaling in CD70<sup>+</sup> 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, CD27 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.<sup>3,4</sup> CD27 stimulation results in recruitment of Siva-1, a poorly understood apoptosis regulatory molecule, and of the adapter protein E3 ligases TRAF2 and TRAF5.<sup>5–8</sup> In accordance with the established role of TRAF2 and TRAF5 in NF $\kappa$ B signaling, CD27 activation results in enhanced activity of the classical and alternative NF $\kappa$ B pathway, which both has been implicated in survival, activation and differentiation of T cells.<sup>9,10</sup>

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.<sup>11–14</sup> 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.<sup>15–18</sup> Furthermore, it has been demonstrated that CD70-secreting tumor cells induce an enhanced anti-tumor immune response.<sup>19,20</sup> 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.<sup>21,22</sup> 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 cancer<sup>23</sup> (<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

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**Abbreviations:** ADCC, antibody-dependent cellular cytotoxicity; GpL, Gaussia princeps luciferase; scFv, single-chain variable fragment; TRAF2/5, TNF receptor-associated factor 2/5; TRAIL, tumor necrosis factor (TNF)-related apoptosis-inducing ligand; TRAILR1/2/3/4, TRAIL receptor-1/2/3/4

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inhibitory effect of the endogenously present serum IgG or 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.<sup>24,25</sup> 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.<sup>26,27</sup> It has been shown that the poor responsiveness of TRAIL death receptors (particular of TRAILR2) toward soluble TRAIL trimers can be overcome in two ways. First, 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.<sup>28</sup> Noteworthy, the latter principle not only allows potent TRAIL death receptor activation but also makes this activation dependent on cell surface antigen binding. Thus, by use of tumor marker-specific scFvs for generation of scFv-TRAIL fusion proteins, tumor-restricted TRAIL death receptor activity can be achieved.<sup>28</sup>

Dulanermin, a recombinant form of soluble TRAIL, has been evaluated in clinical trials and showed a good safety profile but also lack of efficacy.<sup>24,29</sup> Against the background of the limited activity of soluble TRAIL, it appears indeed unlikely that Dulanermin unleashes the full apoptosis-inducing capacity of the TRAIL death receptors. There is a similar situation with TRAILR1- and TRAILR2-targeting antibodies. It has been found that oligomerization or binding to Fc $\gamma$ -receptors (Fc $\gamma$ Rs) strongly enhances the agonistic activity of TRAILR1/2-specific antibodies.<sup>30–35</sup> Most important, *in vivo*, Fc $\gamma$ R1b is especially relevant to unleash the agonistic activity of TRAIL death receptor-specific antibodies.<sup>34,35</sup> The reason for the prominent role of Fc $\gamma$ R1b presumably is based on its favorable expression pattern compared with other Fc $\gamma$ Rs. Now, the TRAILR1/2-targeting antibodies under investigation in clinical trials (Conatumumab, Mapatumumab, Tigatuzumab, PRO957890, CS-1008) are of the IgG1 isotype, which has only a very low affinity for Fc $\gamma$ R1b. The capacity of these antibodies to trigger TRAIL death receptor signaling in patients is therefore probably also low.

Here, we describe the construction of fusion proteins consisting of a llama-derived CD70-specific scFv, and 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<sup>+</sup>-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.<sup>11–14</sup> 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

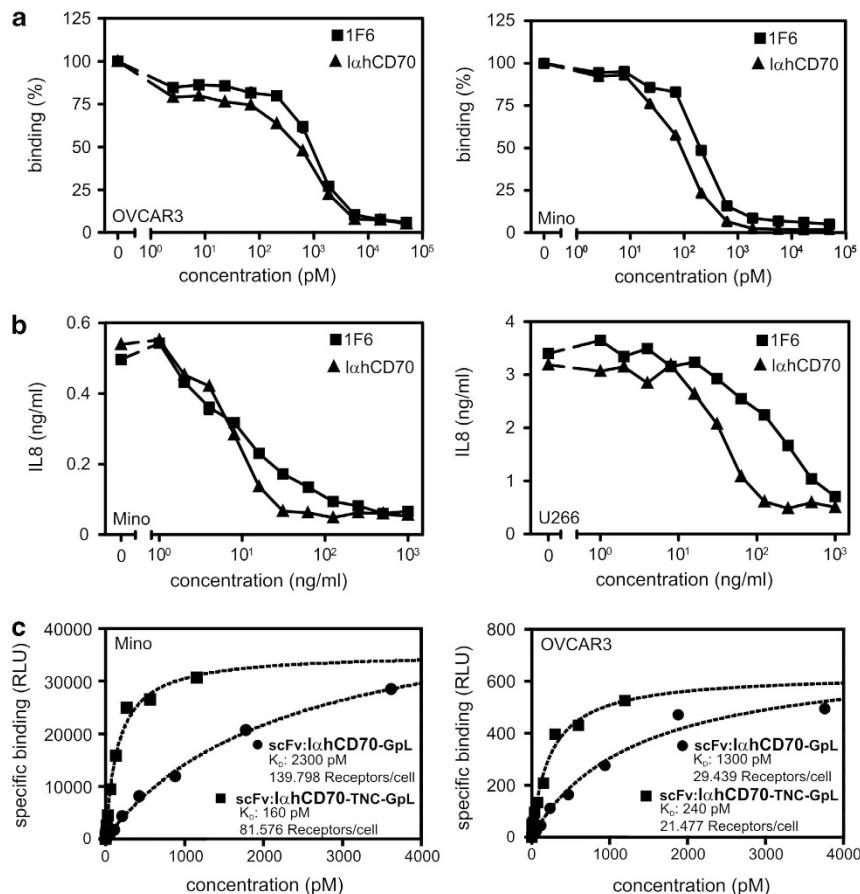
Cell line	Type	CD70	CD27
Raji	NHL	+++	+++
U266	MM	+++	–
Amo-1	MM	+++	–
RPMI	MM	++	–/+
INA6	MM	–	–
KMS-11	MM	++	–
MM1.S	MM	++	–
KMS12.BM	MM	+++	+++
JNN3	MM	+++	–
Mino	MCL	+++	–
Jeko-1	MCL	+++	–
Rec-1	MCL	+++	–
Granta-519	MCL	+++	+
MJ(G11)	CTCL	+++	–
JVM3	CLL	+++	–
Ramos-Ra1	Burkitt	++	+++
BJAB	Burkitt	+++	–/+
Daudi	Burkitt	++	–
SuDHL6	DLBCL	++	–
Panc-1	Pancreatic cancer	+++	+
Panc89	Pancreatic cancer	++	+
PancTu1	Pancreatic cancer	–	–
MiaPaca-2	Pancreatic cancer	–	–
Colo357	Pancreatic cancer	–	–
A498	RCC	+++	–
786-O	RCC	+++	+
FaDu	SCC	–	–
HLaC-78	SCC	–/+	–
WM-35	Melanoma	–/+	–
WM-3211	Melanoma	–	–
WM-115	Melanoma	+++	–
WM-1552C	Melanoma	+/+	–
WM793	Melanoma	–/+	–
WM-3248	Melanoma	++	–
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	Glioblastoma	–/+	–
U87	Glioblastoma	+	–
U343	Glioblastoma	+	–
Ln18	Glioblastoma	–	–

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  $\lambda$ hCD70, from an antibody phage display library constructed from peripheral blood cells of llamas immunized with the human renal cell carcinoma cell line 786-O.  $\lambda$ hCD70 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  $\lambda$ hCD70 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.<sup>36</sup> Activation of CD27 in the HT1080-CD27 cells results in an increase in IL8 production. As expected,  $\lambda$ hCD70 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  $\lambda$ hCD70 with the *Gaussia princeps* luciferase (GpL). Trimerization of scFv: $\lambda$ hCD70 was achieved by fusing the C terminus of the scFv to the trimerization domain of tenascin-C (TNC). The highly traceable GpL domain was furthermore introduced to facilitate binding studies with the scFv: $\lambda$ hCD70 variants. Cellular equilibrium binding studies with the monomeric scFv: $\lambda$ hCD70 protein and the trimeric scFv: $\lambda$ hCD70-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  $\lambda$ hCD70 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.

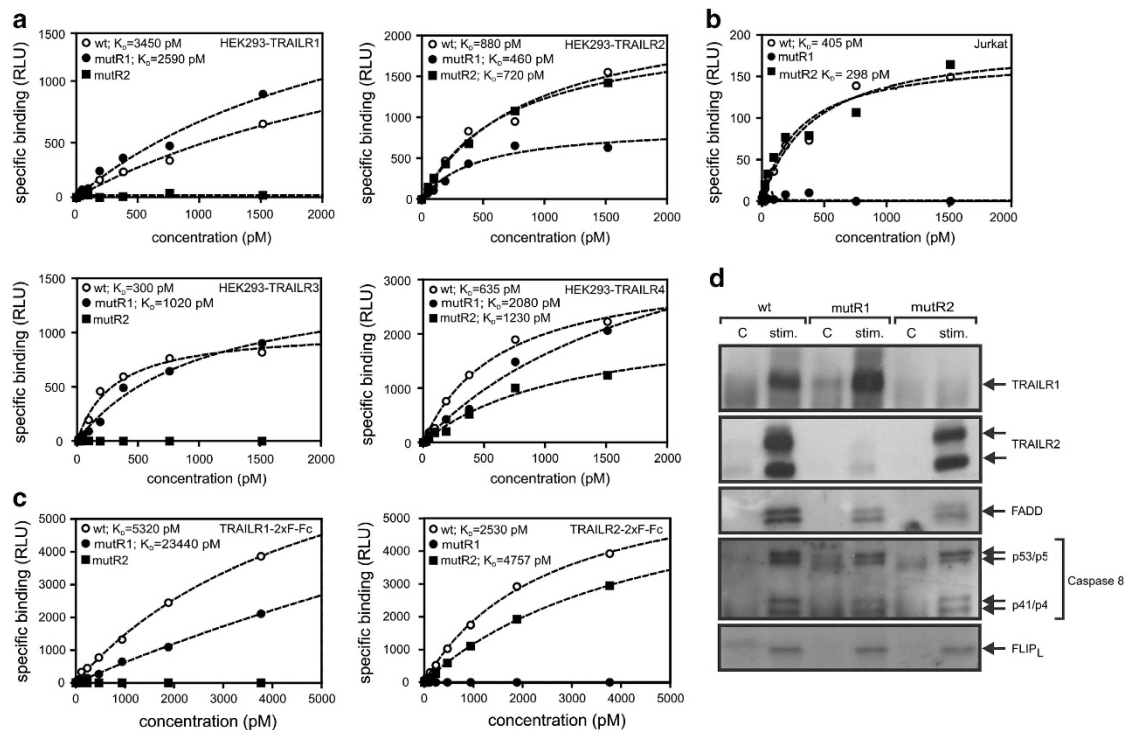
**Characterization of TRAILR1- and TRAILR2-specific TRAIL mutants by cellular binding studies.** It was envisaged in this study to equip a blocking CD70-specific



**Figure 1** CD70-specific mAb  $\lambda$ hCD70 is a potent inhibitor of CD27-CD70 interaction. (a) OVCAR3 and Mino cells were preincubated (1 h) with the indicated concentrations of the CD70-specific mAbs  $\lambda$ hCD70 and 1F6. Cells were then incubated with 200 ng/ml CD27-Fc-GpL for an additional hour and after removal of unbound molecules cell-associated GpL activity was determined. (b) Mino and U266 cells were preincubated (1 h) with the indicated concentrations of the CD70-specific mAbs  $\lambda$ hCD70 and 1F6 and were then used to stimulate IL8 production in HT1080-CD27 cells that were cultured in a 96-well plate. Next day, cell culture supernatants were analyzed for the presence of IL8 by ELISA. (c) Monomeric and trimeric GpL fusion proteins of a  $\lambda$ hCD70-derived scFv were used for equilibrium binding studies with OVCAR3 and Mino cells

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.<sup>37–39</sup> Moreover, there is initial evidence from a recent study that TRAIL death receptor-specific mutants have higher specific activity than the wild-type molecule.<sup>40</sup> Therefore, we introduced recently published mutations in our basic TNC-TRAIL construct conferring

specificity for TRAILR1 (G131R/R149I/S159R/N199R/K201H/S215D) and TRAILR2 (Y189Q/R191K/Q193R/H264R/I266L/D267Q).<sup>40,41</sup> 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 TRAILmutR1 and TRAILmutR2 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

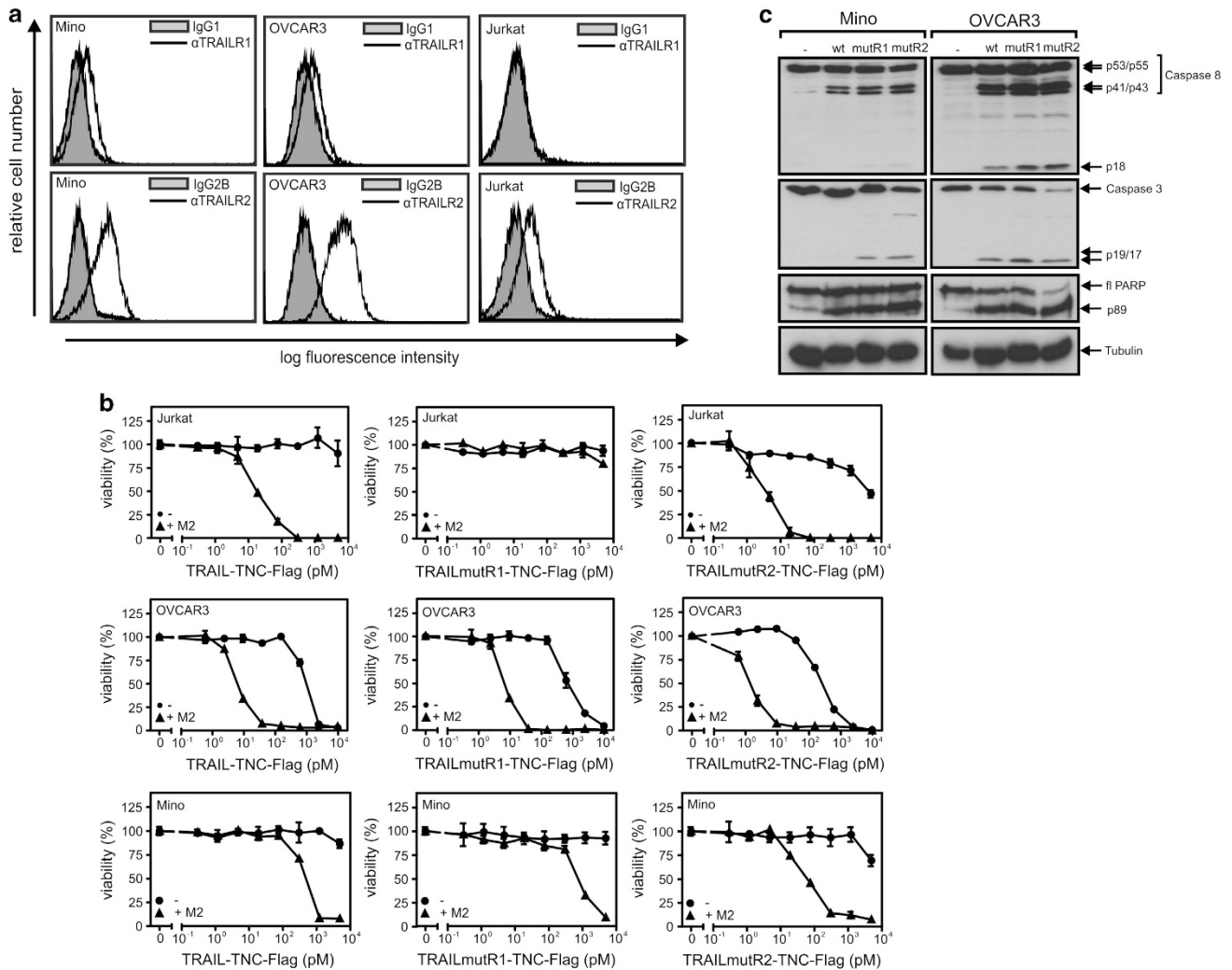


**Figure 2** Analysis of TRAIL receptor binding of TRAILR1 and TRAILR2 discriminating TRAIL mutants. (a) HEK293 cells, which express endogenous TRAILR2 and TRAILR4, were transiently transfected with expression constructs encoding the four types of cell-bound TRAIL receptors. Next day, equilibrium binding studies were performed at 37 °C with GpL-TNC-TRAIL, GpL-TNC-TRAILmutR1 and GpL-TNC-TRAILmutR2. Mock-transfected cells served to determine non-specific binding. (b) Equilibrium binding studies were performed with Jurkat cells, which only express TRAILR2 and the various GpL-TRAIL fusion proteins. Cells pretreated with 5  $\mu$ g/ml TRAIL were used for determination of nonspecific binding. (c) Black 96-well ELISA plates were coated with protein G (0.5  $\mu$ g/ml) and loaded with TRAILR1(ed)-Fc or TRAILR2(ed)-Fc (~1  $\mu$ g/ml) or remained untreated for determination of non-specific binding. After removal of unbound molecules, the indicated concentrations of GpL-TNC-TRAIL, GpL-TNC-TRAILmutR1 and GpL-TNC-TRAILmutR2 were added for 1 h at 37 °C, and finally the well-associated luciferase activity was quantified. (d) HT29 cells were incubated (2 h, 37) with Fc-fusion proteins of the various TRAIL variants and were then immunoprecipitated by help of protein G agarose. Immunoprecipitates and cell lysates were analyzed by western blotting with respect to the presence of the indicated proteins. Lysates of untreated cells were supplemented with 10 ng of the corresponding Fc-fusion protein and served as a negative control

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).<sup>42</sup> 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 huge 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.<sup>26,27</sup> 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,<sup>43</sup> anti-Flag oligomerization of the Flag-tagged TNC-TRAIL variants resulted in a strong increase in cell death induction (Figure 3b).

**scFv:lzhCD70-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 upon cell surface antigen binding.<sup>27,44–49</sup> Thus, scFv-TRAIL fusion proteins showed a prodrug-related behavior, whereby the latently present apoptotic activity of the TRAIL effector molecule is unleashed by sole antigen–scFv domain interaction. The latter reflects that some members of the TNF receptor family are potently activated by the membrane-bound form of their ligand but fail to respond properly to binding of soluble ligand trimers. Indeed, the inability of soluble trimeric TRAIL variants to trigger robust TRAILR2-mediated apoptosis is well established.<sup>26,27</sup> To evaluate the extent to which a prodrug-like activation of TRAILR1 and TRAILR2 can be achieved with CD70-targeted scFv fusion

TRAIL fusion proteins, we constructed derivatives of TNC-TRAIL, TNC-TRAILmutR1 and TNC-TRAILmutR2 bearing N-terminally scFv:lzhCD70 as a CD70-targeting domain (Figures 4a and b). Initially, we transiently expressed CD70 in the CD70-negative HT1080 cell line, which expresses both TRAIL death receptors but mainly signals via TRAILR2 and analyzed the apoptotic effect of the various scFv-TRAIL fusion proteins. scFv:lzhCD70-TNC-TRAIL and scFv:lzhCD70-TNC-TRAILmutR2 but not scFv:lzhCD70-TNC-TRAILmutR1 induced significant cell death in CD70 and mock-transfected HT1080 cells. More important, the ED50-value of scFv:lzhCD70-TNC-TRAIL and scFv:lzhCD70-TNC-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<sup>−</sup> 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:lzhCD70 for CD70 binding. In contrast to the TNC-TRAIL and TNC-TRAILmutR2 variants (Figure 3b), the corresponding scFv:lzhCD70-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:lzhCD70-TNC-TRAIL and scFv:lzhCD70-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 5c). 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:lzhCD70-TNC-GpL, the trimeric variant of the scFv:lzhCD70 antibody fragment already described in Figure 1c. From these experiments, no evidence could be found that CD70 blockade by scFv:lzhCD70-TNC-GpL interferes with cell death induction by TNC-TRAIL or oligomeric TNC-TRAIL (Figure 6a). However, as expected, scFv:lzhCD70-TNC-GpL inhibited the apoptotic activity of our scFv:CD70-TRAIL fusion proteins on CD70<sup>+</sup> cells. Therefore, when the CD70-targeting scFv domain and the TRAIL domain are separated into two distinct molecules



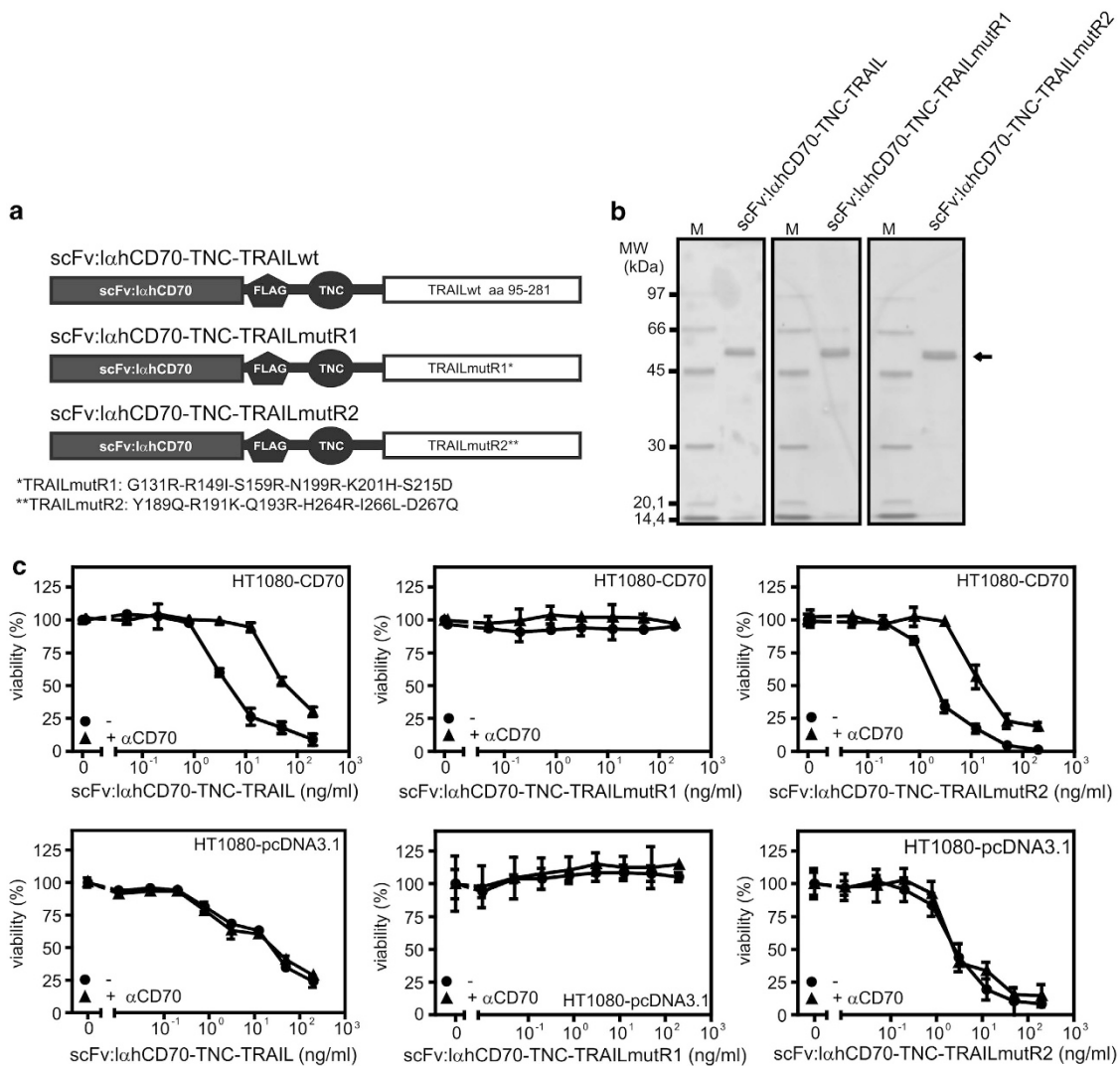
**Figure 3** Apoptosis induction by TRAILR1 and TRAILR2 discriminating TRAIL mutants. **(a)** OVCAR3, Mino and Jurkat cells were analyzed with respect to cell surface expression of TRAILR1 and TRAILR2 by FACS. **(b)** OVCAR3, Mino and Jurkat cells were challenged in triplicates with the indicated concentrations of Flag-TNC-TRAIL, Flag-TNC-TRAILmutR1 and Flag-TNC-TRAILmutR2 in the presence and absence of 1  $\mu$ g/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  $\mu$ g/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  $\mu$ g/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

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:lzhCD70-TNC-GpL is equally effective as our scFv:lzhCD70-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:lzhCD70-TNC-TRAIL fusion proteins. We also evaluated cell death induction by scFv:lzhCD70-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

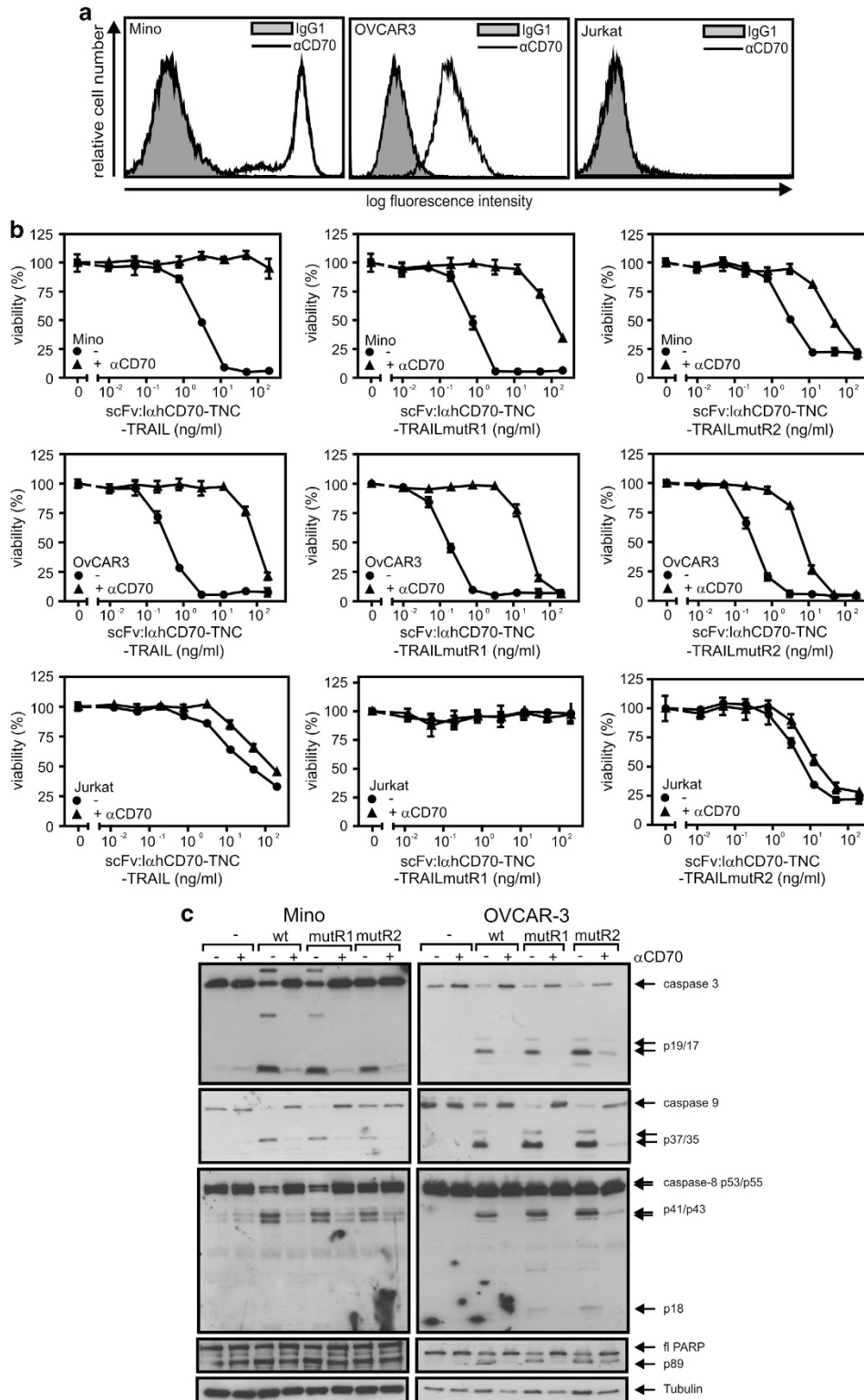
Targeting of CD70 and TRAIL death receptors to treat cancer is currently evaluated in various preclinical studies and phase I clinical trials. In case of CD70 targeting, it was the initial idea to exploit the tumor-associated expression of CD70 to direct antibody-dependent effector functions to tumor 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.<sup>50</sup>



**Figure 4** Initial characterization of scFv:CD70-TRAIL fusion proteins. (a) Scheme of scFv:lαhCD70-TRAIL fusion proteins. scFv:lαhCD70, CD70-specific scFv; F, Flag tag; TNC, tenascin-C trimerization domain; TRAIL, TRAILmutR1 and TRAILmutR2, aa 95–281 of wild-type TRAIL and TRAILR1- and TRAILR2-specific mutants derived thereof. (b) SDS-PAGE analysis of purified scFv:lαhCD70-TNC-TRAIL fusion proteins (100 ng each). (c) HT1080 cells were transiently transfected with a CD70 expression construct or empty vector. Next day, transfected cells were stimulated for an additional day with the indicated concentrations of scFv:lαhCD70-TNC-TRAIL, scFv:lαhCD70-TNC-TRAILmutR1 and scFv:lαhCD70-TNC-TRAILmutR2 in the presence and absence of a competing conventional CD70-specific antibody

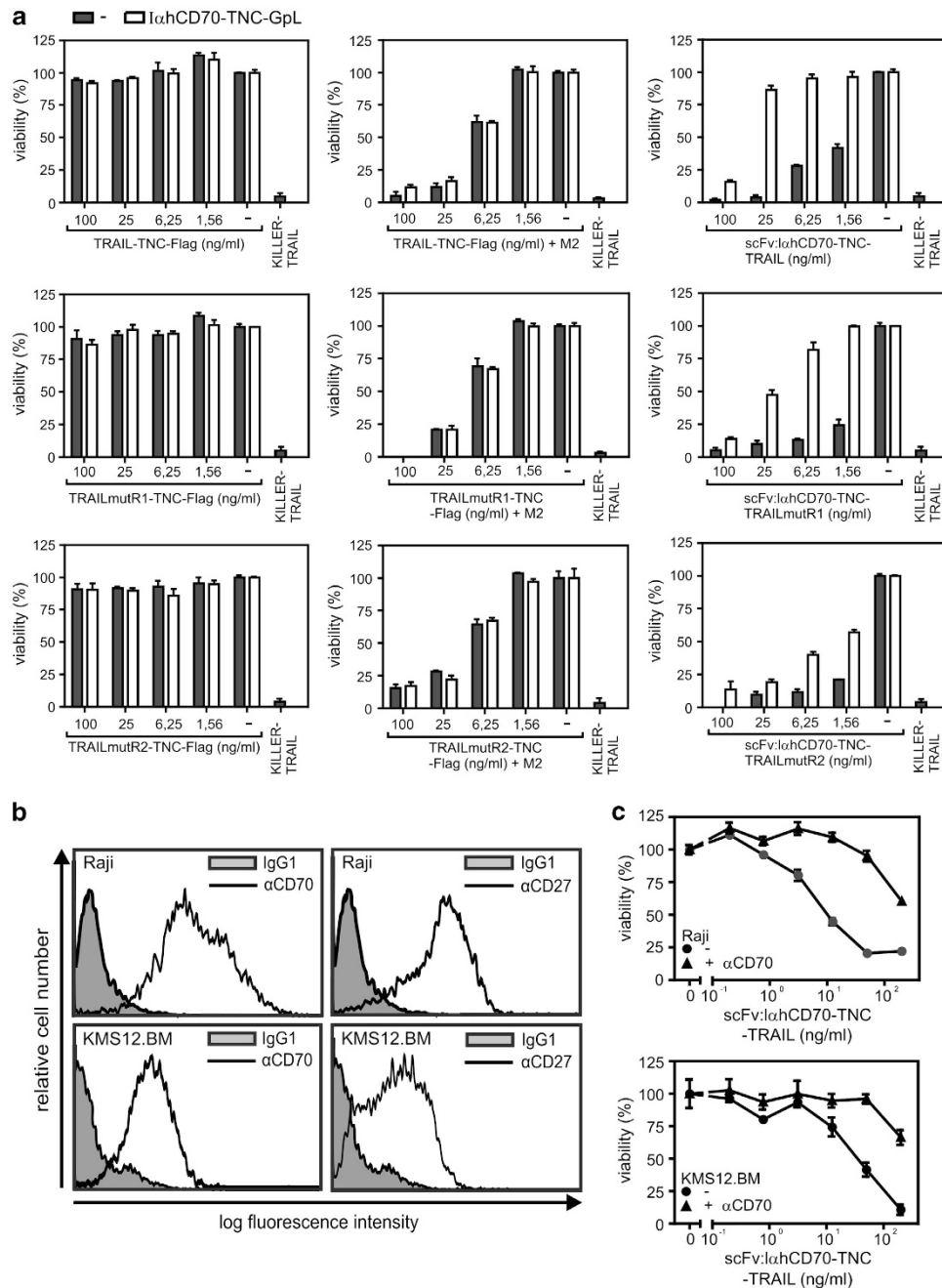
Immunostimulation and cell death induction in tumor, or tumor-associated, cells are two complementary and maybe even synergistic aims in tumor therapy. Combination therapies addressing both of these aims are therefore particularly attractive. To obtain a single molecule that concomitantly allows blockade 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:lαhCD70 (Figure 1). Moreover, in view of former studies, showing superior activity of cell surface antigen-bound TRAIL fusion proteins compared with conventional soluble TRAIL,<sup>27,44–49</sup> we anticipated enhanced cell death induction by the fusion protein when bound to CD70. In fact, the resulting fusion protein, scFv:lαhCD70-TNC-TRAIL, showed the targeted combinatorial 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.<sup>28</sup> To clarify whether both or only one of the two TRAIL death receptors possess improved responsiveness to CD70-bound scFv:lαhCD70-TNC-TRAIL, 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:lαhCD70-TNC-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:λhCD70-TRAIL, scFv:λhCD70-TRAILmutR1 and scFv:λhCD70-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 μg/ml of a scFv:λhCD70 competing conventional CD70-specific antibody. Cells were then challenged overnight in triplicates with the indicated concentrations of scFv:λhCD70-TNC-TRAIL, scFv:λhCD70-TNC-TRAILmutR1 and scFv:λhCD70-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 λhCD70 for 4–6 h with 100 ng/ml of scFv:λhCD70-TNC-TRAIL, scFv:λhCD70-TNC-TRAILmutR1 and scFv:λhCD70-TNC-TRAILmutR2. Total cell lysates were analyzed by western blotting for processing of the indicated proteins





**Figure 6** Blockade of CD70 has no effect on cell death induction by conventional TRAIL. **(a)** Mino cells were cultured in 96-well plates, and half of the cells were pretreated with 5  $\mu$ g/ml of scFv:IαhCD70-TNC-GpL and then challenged overnight in triplicates with the indicated concentrations of TNC-TRAIL, TNC-TRAILmutR1 and TRAILmutR2 with and without anti-Flag (1  $\mu$ g/ml) oligomerization. Cellular viability was determined using the MTT assay. **(b)** FACS analysis of CD70 and CD27 expression of Raji and KMS12.BM cells. **(c)** Raji and KMS12.BM cells were cultured in 96-well plates in the presence and absence of 10  $\mu$ g/ml of a scFv:IαhCD70 competing conventional CD70-specific antibody and were stimulated overnight in triplicates with the indicated concentrations of the various scFv:IαhCD70-TRAIL fusion proteins. Cellular viability was determined using the MTT assay

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<sup>27,28</sup> and the need of anti-TRAIL death receptor antibodies to bind to Fc $\gamma$ R1/IIb to become strongly agonistic *in vivo*,<sup>34,35</sup> 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 Fc $\gamma$ R1/IIb 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.<sup>21,51,52</sup> 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.<sup>53</sup> 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 (PAA, Cölbe, 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 a reach a concentration of 10 ng/ml.

**Molecular cloning, production and purification of recombinant proteins.** A pCR3-based (Invitrogen) expression plasmid encoding for Flag-TNC-TRAIL with an Ig leader sequence has been published elsewhere.<sup>43</sup> To introduce published mutations that allow discrimination of 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 TRAILR1 binding has been reported<sup>41</sup> was designated in the following as TRAILmutR2, and the TRAIL mutant G131R/R149I/S159R/N199R/K201H/S215D for which strongly reduced binding to TRAILR2 has been published<sup>40</sup> was termed TRAILmutR1. To obtain the *Gaussia princeps* luciferase (GpL) fusion proteins of the various TRAIL variants, the Ig leader sequence in the pCR3-Flag-TNC-TRAIL expression constructs was changed with a DNA fragment encoding a humanized version of GpL including the molecules authentic leader sequence. The CD70-specific human IgG1 antibody 1F6 was cloned with the help of a synthetic gene designed according to the published sequences of the variable domains of this antibody (US 2006/0083736A1) and sequences encoding the constant region of human IgG1 (accession number P01857) and the constant domain of the V-J-C segment of the immunoglobulin kappa chain (accession number AAA59000).

To obtain lzCD70 (27B3), a Fab fragment phage library derived from llamas immunized with 786-O cells (ATCC) was screened for antibodies interfering with CD70-CD27 interaction and high affinity for CD70 essentially as described elsewhere.<sup>54</sup> The variable regions of lzCD70 were linked with expression cassettes of the constant regions of the human lambda light chain and the IgG1 region heavy chain. To generate expression plasmids for the scFv: lzCD70-TNC-TRAIL variants, a synthetic gene encoding the Ig leader followed by the VH and VL region of lzCD70 connected by a (GGGG)<sub>3</sub>-linker was used to replace the Ig leader encoding sequence of the corresponding pCR3-Flag-TNC-TRAIL plasmids. The expression plasmid for CD27-Fc-Flag-GpL was obtained by insertion of an expression cassette encoding the extracellular domain of CD27 including leader sequence (aa 1–191) followed by aa 244–469 of hlgG1 (accession number AF019036), a Flag tag and GpL devoid of its leader sequence into the *Hind3* and *Xba1* sites of pCR3. To obtain TRAILR1(ed)-Fc and TRAILR2(ed)-Fc, amplicons encoding the extracellular domains of these proteins were linked to the Fc domain of hlgG1 in the pCR3 expression vector. The various secretable proteins were

transiently produced in Hek293 cells as recently described elsewhere.<sup>55</sup> In brief, 1 ml of suspended Hek293 cells ( $40 \times 10^6$ /ml) were electroporated (250 V, 1800  $\mu$ F, maximum resistance, Easyject Plus electroporator (PepLab, Erlangen, Germany) with 40  $\mu$ g of the corresponding expression plasmid, recovered overnight in cell culture medium with 10% FCS and then further cultivated in 2% FCS-containing medium. After 5–7 days, supernatants were collected, cleared by centrifugation and analyzed with respect to the concentration of the recombinant proteins by anti-Flag western blotting or by assaying luciferase activity. The scFv: lzCD70-TNC-TRAIL fusion proteins were purified by help of their internal Flag tag by affinity chromatography on anti-Flag agarose according to the recommendations of the supplier.

**Equilibrium binding studies.** To measure total binding, cells ( $3\text{--}10 \times 10^5$  cells) were incubated for 1 h with increasing concentrations of the GpL fusion protein of interest at 37 °C. Cells were then washed five times with 1.5 ml icecold PBS in Eppendorf reaction tubes and assayed for cell-bound GpL activity using the *Gaussia* Luciferase Assay Kit (New England Biolabs GmbH, Frankfurt a. M., Germany) and a Lucy 2 Luminometer (Anthos Labtec Instruments, Krefeld, Germany). In case of the equilibrium binding studies with the GpL fusion proteins of the various TRAIL variants and Hek293 cells transiently transfected with the various TRAIL receptors, binding to cells transfected with empty vector was considered as nonspecific binding due to the low expression of the endogenous receptors. In case of equilibrium binding studies with scFv: lzCD70-GpL and scFv: lzCD70-TNC-GpL, cells were preincubated for 1 h with 10  $\mu$ g/ml of conventional competing CD70-specific antibodies to obtain nonspecific binding. Specific binding values were calculated by subtraction of nonspecific binding values from the corresponding total binding values.  $K_D$  values were derived from the specific binding values by nonlinear regression using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). To determine the binding of the various GpL-TRAIL fusion proteins to immobilized TRAIL death receptors, black high bond 96-well ELISA plates were coated with protein G (0.5  $\mu$ g/ml) and subsequently loaded with TRAILR1(ed)-Fc- and TRAILR2(ed)-Fc-containing cell culture supernatants ( $\sim 1$   $\mu$ g/ml) for at least 1 h (RT). Wells were then incubated with increasing concentrations of GpL-TNC-TRAIL, 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. Nonspecific binding data were derived from wells incubated with supernatants of mock-transfected cells instead of TRAILR1/2-Fc.  $K_D$  values were again obtained by fitting the binding data to a one side specific binding plot using GraphPad Prism5.

**Heterologous competition assays with CD27ed-Fc-GpL.** Cells ( $3\text{--}10 \times 10^5$  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 Lucy 2 Luminometer.

**FACS analysis.** Cell surface expression of CD70, TRAILR1 and TRAILR2 was determined by help of CD27 (clone 57703, BD), CD70 (clone Ki-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 FACSCalibur (BD Biosciences, Heidelberg, Germany).

**Cell death assay.** Cells were seeded in 96-well plates ( $10 \times 10^3$ /well for adherent cell lines and  $50 \times 10^3$ /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° 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 declare no conflict of interest.

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