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ORIGINAL ARTICLE

TRAIL signaling is mediated by DR4 in pancreatic tumor cells despite the expression of functional DR5

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Abstract Tumor necrosis factor related apoptosis-inducing ligand (TRAIL) and agonistic anti-DR4/TRAIL-R1 and anti-DR5/TRAIL-R2 antibodies are currently under clinical investigation for treatment of different malignancies. TRAIL activates DR4 and DR5 and thereby triggers apoptotic and non-apoptotic signaling pathways, but possible different roles of DR4 or DR5 in these responses has poorly been addressed so far. In the present work, we analyzed cell viability, DISC formation as well as IL-8 and NF–κB activation side by side in responses to TRAIL and agonistic antibodies against DR4 (mapatumumab) and against DR5 (lexatumumab) in pancreatic ductal adenocarcinoma cells. We found that all three reagents are able to activate cell death and pro-inflammatory signaling. Death-inducing signaling complex (DISC) analysis revealed that mapatumumab and lexatumumab induce formation of homocomplexes of either DR4 or DR5, whereas TRAIL additionally stimulated the formation of heterocomplexes of both receptors. Notably, blocking of receptors using DR4- and DR5-specific Fab fragments indicated that TRAIL exerted its function predominantly via DR4. Interestingly, inhibition of PKC by Goe6983 enabled DR5 to trigger apoptotic signaling in response to TRAIL and also strongly enhanced lexatumumab-mediated cell death. Our results suggest the existence of mechanisms that silence DR5 for TRAIL- but not for agonistic-antibody treatment.

Keywords TRAIL · DR4/DR5 · Mapatumumab · Lexatumumab · Pancreatic adenocarcinoma

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Introduction

The observation that the death ligand TRAIL (tumor necrosis factor related apoptosis-inducin ligand) kills a variety of tumor cell lines, leaving most normal, healthy cells viable, has raised expectations that it could function as a specific anti-cancer therapeutic weapon [1].

TRAIL induces cell death by activation of two death domain-containing receptors DR4/TRAIL-R1 [2, 3] and DR5/TRAIL-R2/TRICK [4–6]. DR4 and DR5 are highly homologous, and it is still not known if they are fully redundant or are capable of transducing distinct physiological functions. The best understood function of these receptors is their ability to induce apoptosis [7]. Binding of TRAIL to its receptor(s) induces the formation of the death-inducing signaling complex (DISC) which involves the activation of the initiator caspase 8/10. Activated caspase 8/10 subsequently cleaves Bid and/or caspase 3 and initiates the mitochondrial apoptotic pathway and the caspase cascade, respectively, eventually leading to cell death [7].

Many primary tumor cells as well as established tumor cell lines developed mechanisms that allow them to escape

TRAIL-mediated apoptosis [8, 9]. The interplay between pro-apoptotic and anti-apoptotic proteins present in a particular cell type determinates the outcome: cell death or survival. Actually, each step in apoptotic TRAIL signaling can be inhibited by anti-apoptotic proteins such as: DcR1 and DcR2, cFLIP-isoforms, anti-apoptotic Bcl-2-family members, and IAP-family members [7]. Mutations or downregulation of apoptosis-transducing proteins, especially the death receptors [10–12] or caspase 8 [13, 14] may also inhibit TRAIL-mediated cell death in some tumor cells.

TRAIL and its death receptors are also able to activate signaling pathways leading to the activation of NF (nuclear factor) –κB, PKCs (protein kinase C), MAPK, and AKT [7, 15–18].

Despite tremendous efforts, pancreatic ductal adenocarcinoma (PDAC) remains therapy resistant with the death rate almost equal to the incidence rate. The promising effects of TRAIL on different tumor cells prompted us to study the effects of TRAIL on PDAC cells under in vitro and in vivo conditions. We found that the majority of PDAC cells are resistant to TRAIL-mediated apoptosis mainly because of the constitutive up-regulation of anti-apoptotic proteins such as Bcl-xL, FLIP, XIAP, and TRAF2 [19–21]. Moreover, PDAC cells respond to TRAIL with activation of non-apoptotic, pro-inflammatory signaling pathways resulting in activation of NF–κB, PKC, and ERK1/ERK2 [15, 18]. Accordingly, apoptosis-resistant PDAC cells respond to TRAIL with the expression of pro-inflammatory and invasion-promoting proteins and show invasive growth in vitro as well as in vivo in an orthotopic SCID-mouse model of pancreatic cancer [22]. On the other hand, we also showed that TRAIL-mediated apoptosis could be restored by co-treatment of cells with inhibitors of NF–κB or PKC [15]. Thus, the therapeutic usage of TRAIL in treatment of pancreatic adenocarcinoma requires combination with sensitizing drugs.

One way to exploit TRAIL signaling is the specific stimulation of either DR4 or DR5 using agonistic, receptor-specific antibodies. The best studied antibodies to date are mapatumumab and lexatumumab that target DR4 and DR5, respectively [23]. The specific and potent apoptosis-promoting activity of these antibodies has been shown for numerous different tumor cells [23], but, to our knowledge, no real side by side comparison of apoptotic vs. non-apoptotic properties of these antibodies with analogous effects of TRAIL have been described. Keeping in mind the disastrous prognosis of PDAC and the dichotomy of TRAIL activities, we compared the responses of PDAC cells to recombinant TRAIL, mapatumumab and lexatumumab with regard to apoptotic/non-apoptotic signaling and further dissected the DR4 and DR5-mediated TRAIL effects by specific inhibition of either DR5 or DR4.

Materials and methods

Cell culture and viability assays

Human pancreatic adenocarcinoma cell lines Colo357, Panc89, and PT45 have been cultured as described [20]. For stimulation experiments, TRAIL (R&D Systems, Wiesbaden, Germany), mapatumumab and lexatumumab (Human Genome Sciences, Rockville, MD, USA), JNK-inhibitor II (Calbiochem, Bad Soden, Germany), UO126 (Promega, Mannheim, Germany), or Goe6983 (Calbiochem) were used. Cell viability was determined by crystal violet staining [18]. To study the cell death of Jurkat cell, cells were seeded in six-well plates at 5×105 cells/well. Following treatment, cells were collected by centrifugation and resuspended in PBS (phosphate-buffered saline) / 5 mM EDTA (ethylene diamine tetra-acetate) containing 2 μg/ml propidium iodide (PI). Finally, the red fluorescence was measured on a FACSCalibur flow cytometer (BD Biosciences). Data mean±SD (n=3).

Fluorescence-activated cell sorting analysis

The detection of cell surface expression of death receptors was performed as described [20]. Anti-DR4, anti-DR5, anti-DcR1, and anti-DcR2 were purchased from Alexis, Heidelberg, Germany.

IL-8 ELISA

Cells were seeded (1×104/well in a 96-well plate), grown for 24 h, and stimulated for additional 16 h with TRAIL, mapatumumab, or lexatumumab. For inhibitory studies DR4- and DR5-specific Fab preparations (1:10), UO126 (10 μM), Goe6983 (20 μM), or JNK-inhibitor II (20 μM) were added to the medium 30 min prior to the exposition to TRAIL. For determination of the IL-8 concentration in culture supernatants, an IL-8-Immunoassay (R&D Systems) was used according to the manufacturer's recommendations.

siRNA studies

siRNAs were purchased from Dharmacon (Thermo Scientific, Schwerte, Germany). Panc89 cells were transfected for 40 h with siRNA specific for DR4 and DR5 or with control siRNA at a final concentration of 75 nM using NeoFX (Ambion, Applied Biosystems, Darmstadt, Germany) according to the manufacturer's protocol.

Western blot analysis

Western blot analysis was performed as described [15]. Primary antibodies used were purchased from: BD Transduction Laboratories, Heidelberg, Germany (anti-

FADD); Cell Signaling, Frankfurt, Germany (anti-DR5 and anti-caspase 8); Chemicon, Milipore, Schwalbach, Germany (anti-DR4, anti-DcR1, and anti-DcR2); and Sigma (anti-β-actin).

Biotinylation of human TRAIL

The extracellular domain of human TRAIL (amino acids 95-281) was biotinylated using Killer-TRAIL preparations (Axxora, Lörrach, Germany) prepared in 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4, 300 mM sodium chloride, 1% sucrose, 0.01% Tween 20, and 1 mM DTT at a concentration of 0.5 mg/ml, supplemented with Na2CO3 (final concentration of 0.1 M). The coupling reaction was started by the addition of a fivefold molar excess of 6-((6-((biotinoyl)amino)hexanoyl)amino)hexanoic acid, succinimidyl ester (biotin-XX, SE; Invitrogen, Karlsruhe, Germany) over TRAIL monomers. After 2 h incubation at RT, protein was separated from non-reacted biotin-coupling reagent by separation on ProSpin (Princeton Separations, NJ, USA) gel filtration spin columns according to the manufacturer's recommendations. The degree of biotin labeling of TRAIL was quantified using a FluoReporter biotin quantification assay kit (Invitrogen) according to the manufacturer's instructions. The biological activity of biotinylated TRAIL on Panc89 cells was similar to unlabeled TRAIL assayed in a cytotoxicity assay (data not shown).

Preparation and analysis of TRAIL receptosomes

TRAIL receptor-containing magnetic membrane fractions were prepared as described for TNF-receptosomes [24]. Briefly, TRAIL receptors were magnetically labeled using biotinylated TRAIL and 50 nm MACS streptavidin-microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were incubated in a total volume of 250 µl cold DMEM with 100 µl (400 ng) of biotin-TRAIL for 1 h at 4°C, followed by incubation with 200 µl MACS streptavidin-microbead solution for 1 h at 4°C. Formation of magnetized TRAIL/TRAIL-receptor complexes were achieved by incubation at 37°C for the time periods indicated in the Fig. 5a and stopped by chilling to 4°C. Cells were collected by centrifugation at 300×*g* and washed with a sucrose buffer containing 0.25 M sucrose, 0.015 M HEPES, 100 mg/l MgCl2, pH 7.4, and Complete Protease Inhibitors Cocktail (Roche Diagnostics, Mannheim, Germany). Cells were mechanically homogenized with glass beads in 250 µl supplemented sucrose buffer at 4°C and postnuclear supernatants containing intact membrane vesicles were subjected to magnetic separation of TRAIL receptor-containing fractions in a high-gradient magnetic field. Samples were finally separated by SDS-PAGE and analyzed by Western blotting using antibodies against DR4 (Chemicon), DR5 (Cell Signaling), FADD (BD Transduction Laboratories), and caspase-8 (gift from M.E. Peter, Chicago, C-15, [25]).

Immunoprecipitation

One subconfluent 175-cm2 flask of Panc89 cells was used per condition. Cells were stimulated with either TRAIL (100 ng/ml; R&D Systems) or with mapatumumab or lexatumumab (both 10 µg/ml) for 1 h at 37°C or left untreated. Cells were washed twice with ice-cold PBS and lysed with 1.5 ml lysis buffer (30 mM Tris–HCl, pH 7.5, 1% Triton X-100, 10% glycerol, and 120 mM NaCl) supplemented with complete protease inhibitor cocktail (Roche) for 20 min on ice. After centrifugation (30 min, 14,000×*g*) mapatumumab/lexatumumab associated proteins were precipitated with protein G sepharose beads (Roche) overnight at 4°C. Lysates of TRAIL-stimulated cells, and untreated control samples were either supplemented with 10 µg mapatumumab or 10 µg lexatumumab prior to precipitation with protein G beads. The precipitates were washed five times with ice-cold lysis buffer. Bound proteins were eluted by incubation at 70°C for 10 min in Laemmli buffer.

Generation of DR4- and DR5-specific Fab fragments

DR4- and DR5-specific antisera were obtained by custom immunization of rabbits (Eurogentec, Seraing, Belgium) with purified DR4-Fc and DR5-Fc (kind gift from Pascal Schneider, University of Lausanne, Switzerland). Antibodies were purified using 5 ml HiTRAP rProtein A FF columns (GE Healthcare, Munich, Germany) according to the manufacturer's protocol. The specificities of antisera were proven on KB cells expressing both DR4 and DR5 and on Jurkat cells expressing only DR5. Both antisera were able to kill KB cells, whereas only antiserum raised against DR5 led to death of Jurkat cells (data not shown). As it has been shown especially for CD95 that Fab fragments in contrast to complete agonistic antibodies inhibit death receptor signaling [26], we generated Fab fragments from these antisera using the Fab Preparation Kit from Pierce (Bonn, Germany). The obtained Fab-containing fractions were finally dialyzed against distilled water and quantified via Western blotting.

Electrophoretic mobility shift assay

Nuclear extracts were prepared as described [27]. Electrophoretic mobility shift assays (EMSAs) were performed by analyzing 5 µg of nuclear extract with the Gelshift NF–κB family (Carcinoma) kit (Active Motif, Rixensart, Belgium) following manufacturer's instructions. For supershift assay,

0.4 μg of anti-p65 or 0.4 μg of anti-p50 antibodies (Santa Cruz) were added to the sample and incubated for the additional 1 h at 4°C. The samples were separated on native 6% polyacrylamide gels in low-ionic strength buffer (0.25× Tris–borate–EDTA) and visualized by autoradiography.

Results

TRAIL induces cell death in PDAC cells via DR4

Previously, we have shown that PDAC cells respond to exogenous TRAIL with cell death and non-apoptotic signaling in parallel. Since TRAIL and different agonistic anti-TRAIL-receptor antibodies are currently evaluated in clinical trials, we performed studies to characterize the receptor-specific signal transduction in PDAC cells.

First we carried out fluorescence-activated cell sorting (FACS) analyses of TRAIL-receptor expression on the cell surface of Colo357, Panc89, and PT45 cells. As shown in Fig. 1a, all cell lines expressed the death-inducing TRAIL receptors, DR4 and DR5.

Since it has been shown that decoy receptors DcR1 and DcR2 may inhibit TRAIL-mediated apoptosis [7], we also determined the cell surface expression of these TRAIL receptors. We found that Panc89 and PT45 cells showed significant cell surface expression of DcR2 (Fig. 1a) while only modest amounts of this receptor was present on the cell surface of Colo357. DcR1 was almost undetectable on the plasma membrane of cell lines studied. Absence of noticeable cell surface expression of the TRAIL decoy receptors was due to post-transcriptional regulation as both decoy receptors could be detected intracellularly by Western blot analyses of whole cell lysates (Fig. 1b). Western blot analysis of DR5 expression showed three immunoreactive bands of about 32, 40, and 48 kDa. To prove that all these bands represent DR5, we performed knockdown experiments using siRNA targeting DR5. As shown in Fig. 1c, siRNA against DR5 strongly reduced the immunoreactivity of not only the two larger bands that corresponded in size to two known alternatively spliced variants of DR5, but also the intensity of the smallest band. Specific downregulation of DR4 did not change the intensity of any of the three anti-DR5 reactive bands.

Next, we determined the viability of cells treated with different concentrations of TRAIL (Fig. 2a). We found that Colo357 cells were slightly more sensitive to TRAIL than Panc89 and PT45 (Fig. 2a). These results did not correlate with the expression levels of DR4 and DR5 at the cell surface since Panc89 and PT45 cells expressed more DR5 than Colo357.

To analyze the functionality of both TRAIL death receptors, we targeted each receptor directly using the highly specific agonistic anti-TRAIL-R1 (DR4) and anti-

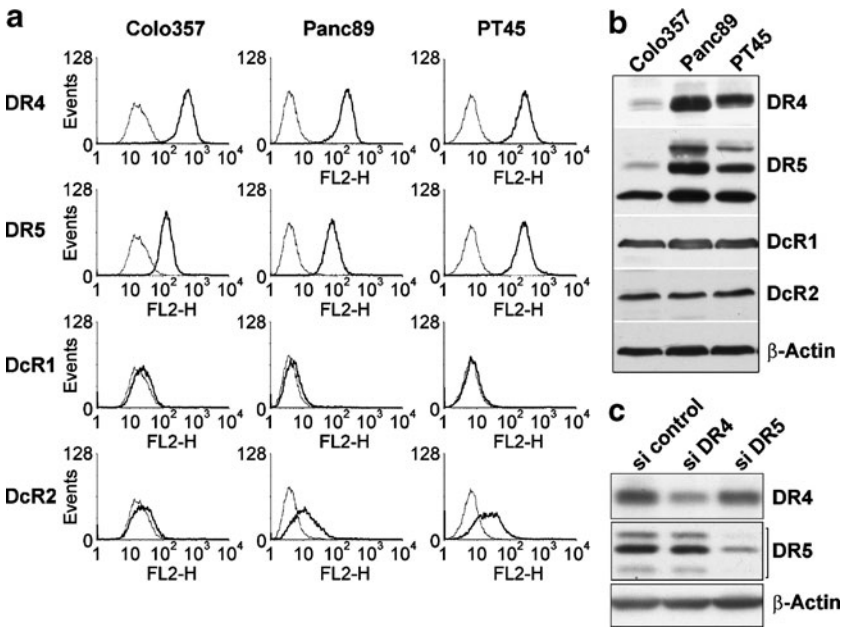

Fig. 1 Expression of TRAIL receptors in PDAC cells. **a** Flow cytometric analysis of cell surface expression of DR4, DR5, DcR1, and DcR2 on Colo357, Panc89, and PT45 cells. *Bold lines*: specific monoclonal antibodies as indicated in the figure, *thin lines* corresponding isotype control (IgG1). **b** Western blot analysis showing the expression of TRAIL receptors in whole cell lysates of cells. β-actin detection served as a loading control. **c** Downregulation of DR4 and DR5 by siRNA. Panc89 cells were transfected with DR4-siRNA, DR5-siRNA, or control siRNA. After 40 h, whole cell lysates were prepared with RIPA buffer. Expression of DR4 and DR5 was determined by Western blot with β-actin as loading control

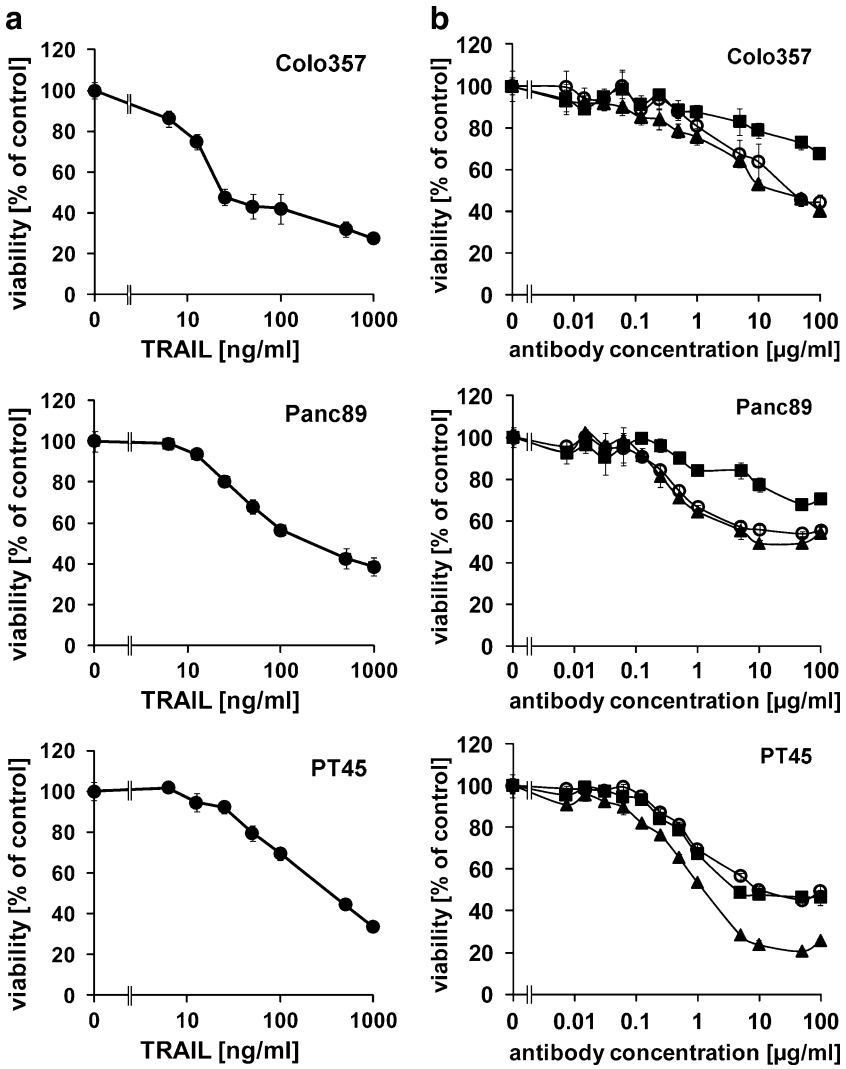

Fig. 2 Sensitivity of PDAC cells to TRAIL and agonistic receptor-specific antibodies. Colo357, Panc89, and PT45 cells were treated with the indicated concentrations of TRAIL (**a**) or mapatumumab/lexatumumab (**b**); *open circle*, mapatumumab, *filled square* lexatumumab, *filled triangle* mapatumumab+lexatumumab. After 16 h, cell viability was determined by crystal violet staining. Shown are results from one representative experiment out of three performed. Data show means±SD (n=4)

TRAIL-R2 (DR5) antibodies, mapatumumab and lexatumumab, respectively, that are currently under clinical trials for treatment of various malignancies [9, 23]. We performed dose response experiments (Fig. 2b) and found that both, mapatumumab and lexatumumab, induced cell death. Only in one cell line, PT45 concomitant treatment of cells with both agonistic antibodies further enhanced cell death (Fig. 2b).

We further tested the relevance of each of the two TRAIL death receptors for TRAIL-induced apoptosis. To do so, we generated Fab fragments from polyclonal rabbit anti-DR4 and anti-DR5 antisera for blocking experiments. Using these reagents, we found that TRAIL-signaled cell death in PDAC cells predominantly via DR4 (Fig. 3) irrespective of the fact that these cells express both, DR4 and DR5 at the cell surface (Fig. 1) and that both receptors are able to induce cell death when targeted with agonistic antibodies (Fig. 2b). This was also not due to DR4-limited TRAIL activity since the TRAIL preparation used in our experiments efficiently induced apoptosis in Jurkat cells, which express only DR5 at the cell surface (Fig. 4a, b). Of note, the DR5-targeting Fab fragments used in our study were highly potent in blocking TRAIL-mediated apoptosis of Jurkat cells (supplementary Fig. 1).

For further characterization of apoptotic TRAIL signaling, we stimulated Panc89 cells with biotinylated TRAIL and analyzed TRAIL-induced DISC formation by isolation of TRAIL receptor-containing vesicles using streptavidin-coated magnetic nano-beads and a custom-built magnetic separation system [24]. As shown in Fig. 5a, the 52/55 kDa procaspase-8 is converted into the 41/43 kDa receptor-bound cleaved caspase-8 within 10 min, indicating activation of caspase-8 by autoproteolysis. A slight increase of the 18-kDa band representing the other active caspase-8 cleavage product is observed in parallel, with the prominent increase of this band appearing after 60 min. Both, DR4 and DR5 were bound to TRAIL, but, using this approach, it was not possible to determine which receptor

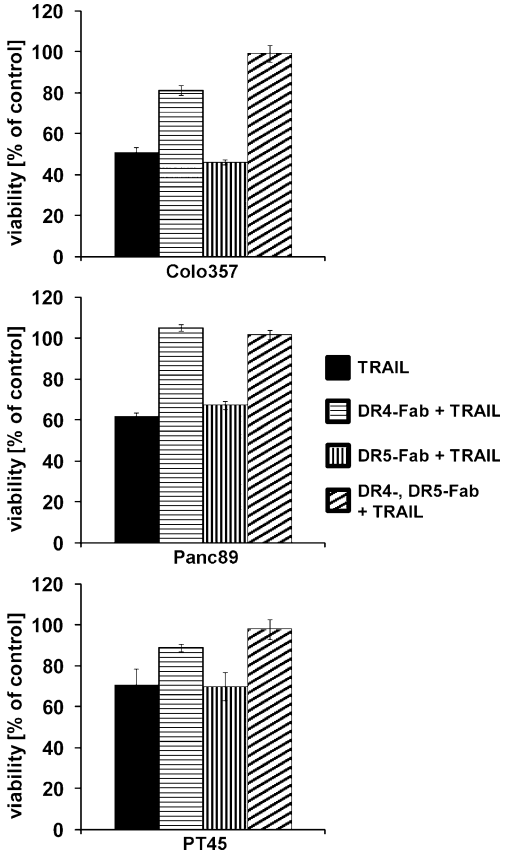

Fig. 3 In PDAC cells, TRAIL signals cell death exclusively via DR4. Cells were treated with 100 ng/ml TRAIL for 16 h with or without antagonistic, receptor-specific antibodies (DR4-Fab or DR5-Fab) that were added to the cell culture 1 h prior to TRAIL. Cell death was determined by crystal violet staining. Data show mean values±SD (n=4)

was responsible for capase-8 activation and thus for apoptosis induction. Moreover, the question whether TRAIL has formed DR4 and DR5 hetero- or homomeric-complexes remained to be answered. To address these questions, we further analyzed the DISC composition in TRAIL-stimulated Panc89 cells using mapatumumab and lexatumumab for immunoprecipitation. In parallel, we used mapatumumab and lexatumumab for cell stimulation and, after cell lysis, isolated the formed complexes by immunoprecipitation without addition of any further antibodies. Analyses of the cell lysates used for the immunoprecipitation demonstrated that TRAIL as well as mapatumumab and lexatumumab induced cleavage of caspase-8 (Fig. 5b). Furthermore, in accordance with the viability data shown in Fig. 2b, mapatumumab treatment led to more effective caspase-8-cleavage than lexatumumab (Fig. 5b). Analyses of proteins that co-immunoprecipitated with DR4 or DR5 revealed that following stimulation of cells with agonistic antibodies, the caspase-8 processing intermediate p41/43 could be detected in both the DR4-associated and the DR5-associated DISC (Fig. 5c lanes 3 and 4). In contrast, the caspase-8 processing intermediate was only detectable in DR4-immunoprecipitates of TRAIL-stimulated cells (Fig. 5c, lane 5), but was absent from the TRAIL–DR5 complexes (Fig. 5c, lane 6).

The immunoprecipitation experiments further revealed that mapatumumab and lexatumumab stimulation led to formation of homomeric receptor complexes with either DR4 or DR5, respectively (Fig. 5c, lanes 3 and 4). Neither DR5 could be detected in DR4-complexes nor DR4 in DR5-complexes. On the contrary, TRAIL clearly induced the formation of heteromeric complexes of DR4 with DR5 since antibody used for precipitation of DR4 from lysates of TRAIL-treated cells precipitated DR4 and DR5 and conversely, antibody against DR5 precipitated not only DR5 but also DR4 (Fig. 5c, lanes 5 and 6). No DcR1 or DcR2 could be detected in immunoprecipitates of DR4 or DR5, neither in untreated control cells nor in TRAIL or mapatumumab- or lexatumumab-treated cells (data not shown). Interestingly, TRAIL recruited the 32 kDa form of DR5 (Fig. 5a, c, lane 6) whereas recruitment of this form neither occurred with mapatumumab nor with lexatumumab (Fig. 5c, lane 3 and 4).

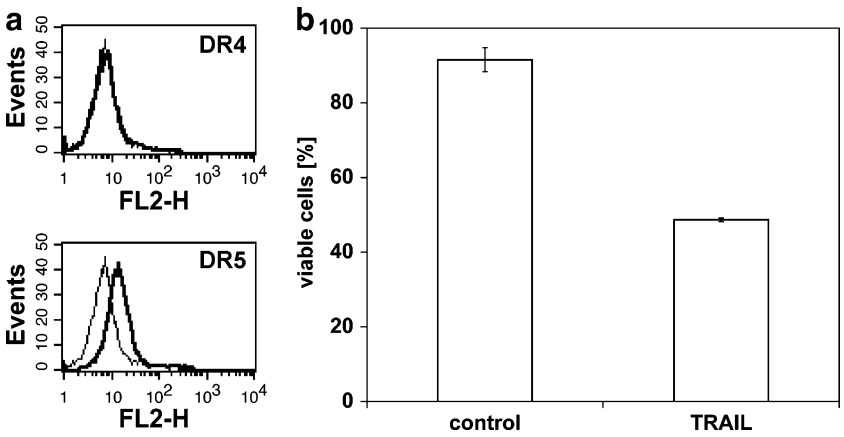

Fig. 4 Jurkat cells express only DR5 at the cell surface and are sensitive to TRAIL. **a** Flow cytometric analysis of the expression of DR4 and DR5 at the cell surface of Jurkat cells. **b** Jurkat cells were treated for 16 h with 100 ng/ml of TRAIL. Cell death was determined by FACS analysis of propidium iodide stained cells (n=3)

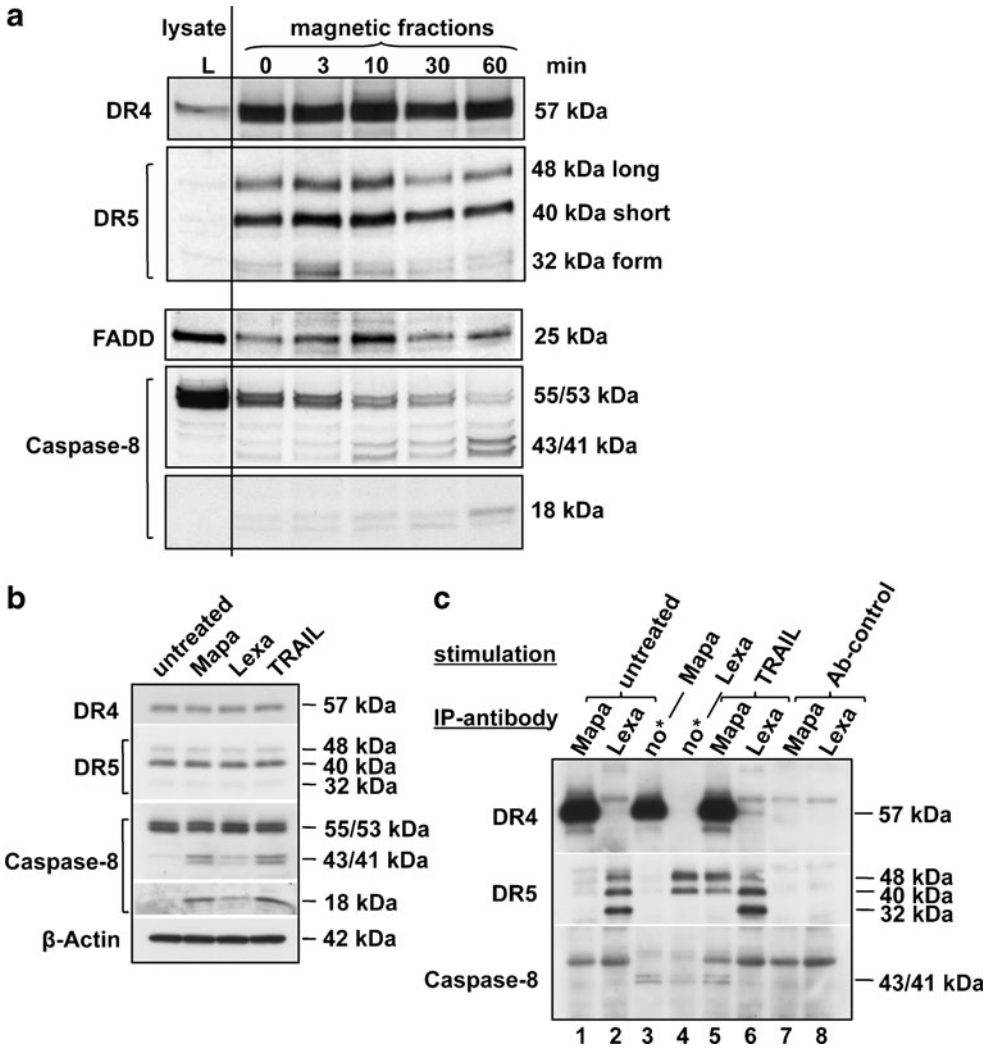

Fig. 5 DISC analyses of TRAIL, mapatumumab-, and lexatumumab-stimulated cells. **a** Magnetic fractions harboring labeled TRAIL/TRAIL receptor complexes were purified from Panc89 cells using a high magnetic gradient chamber and immunoblotted with the antibodies indicated. Cellular whole protein extracts were used as expression controls (lysate). Both, DR4 and DR5 are detected, indicating binding of biotinylated TRAIL to both receptors. FADD is recruited and caspase-8 is activated in these fractions. **b**, **c** Panc89 cells were treated for 1 h with mapatumumab (10 μg/ml), lexatumumab (10 μg/ml), or TRAIL (100 ng/ml) and lysed (section Materials and methods). **b** The lysates were analyzed by Western blot for the expression of DR4, DR5, and caspase-8. As a loading control β-actin expression levels were determined in parallel. **c** DR4 or DR5 were precipitated from lysates using mapatumumab or lexatumumab as described in Materials and methods. For antibody-induced DISC (*lanes 3* and *4*), stimulation antibody was utilized for IP and no further antibody was added (*no**). The immunoprecipitates were resolved in 4–20% Tris–glycine gels and analyzed for DR4, DR5, and caspase-8 using Western blotting

TRAIL induces non-apoptotic signaling predominantly via DR4, but both receptors are able to induce non-apoptotic signaling when stimulated by agonistic antibodies

To specify the role of DR4 and DR5 in non-apoptotic TRAIL signaling in PDAC cells, we performed studies on PDAC cells using the mapatumumab and lexatumumab antibodies as well as recombinant TRAIL alone and in conjunction with DR4- and DR5-specific Fab fragments. As a readout, we analyzed the secretion of IL-8. Both, DR4 and DR5, when stimulated separately by agonistic antibodies, were able to induce secretion of IL-8 (Fig. 6a). Similar to the apoptotic response, stimulation of DR4 resulted in stronger induction of IL-8 secretion than stimulation of DR5. Likewise, treatment of PDAC cells with TRAIL resulted in strong induction of IL-8 secretion (Fig. 6b). Interestingly, much lower concentrations of TRAIL were needed for IL-8 induction (Fig. 6b) as for the induction of cell death (Fig. 2a). Importantly, TRAIL-induced IL-8-secretion was almost completely blocked in the presence of DR4-specific Fab fragments, but was only partially diminished (PT45), remained unchanged (Colo357), or was even enhanced (Panc89) when DR5 was blocked (Fig. 7a). Simultaneous blocking of both DR4

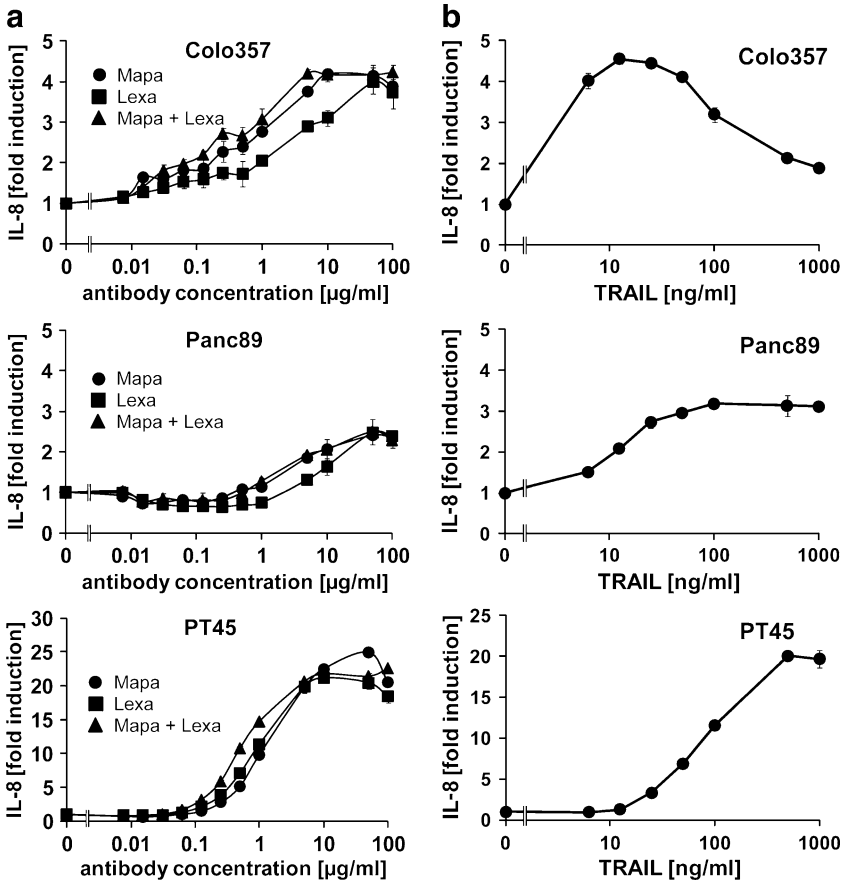

Fig. 6 Mapatumumab, lexatumumab, and TRAIL all induce the secretion of IL-8 in PDAC cells. Colo357, Panc89, and PT45 cells were treated with different concentrations of agonistic antibodies (**a**) mapatumumab, lexatumumab, combination of both or with TRAIL (**b**) for 16 h. The concentrations of IL-8 in cell culture supernatants were determined by ELISA. Data represent means±SD (n=4)

and DR5 totally abolished TRAIL-mediated IL-8 secretion. Since NF–κB activation is of crucial relevance for IL-8 expression, we also determined TRAIL-receptor-induced DNA binding of NF–κB in Panc89 cells (Fig. 7b).

We found that both receptors, DR4 and DR5, were able to efficiently trigger NF–κB binding to a consensus NF–κB site. Similarly, stimulation of cells with TRAIL-induced efficient DNA binding. Supershift assays showed that the upper band, which was predominantly induced by TRAIL- and agonistic-antibody treatment, represents the p65/p50 and the lower band the p50/p50 dimer (data not shown). As expected from the data analyzing IL-8 induction, activation of NF–κB following TRAIL stimulation was driven by DR4, and inhibition of DR5 did not inhibit (1 h) or even slightly enhance (3 h) TRAIL-induced NF–κB activation (Fig. 7b).

Goe6983 sensitizes DR5 to TRAIL-induced cell death

To verify if DR5 could be "re-activated" for TRAIL-induced cell death, we pre-treated cells with inhibitors of different, potentially anti-apoptotic signal transduction pathways and subsequently exposed them to TRAIL in the presence or absence of DR4- or DR5-specific Fabs. We found that inhibition of PI-3/Akt-Kinase and JNK did not influence cell viability following TRAIL treatment (data not shown). Interestingly, the MEK-inhibitor UO126 strongly sensitized Panc89 cells to TRAIL-induced cell death, but mainly DR4-signaling was enhanced (Fig. 8a). More importantly, Goe6983, an inhibitor of PKCs, not only sensitized cells to TRAIL-mediated apoptosis via DR4, but also re-activated the otherwise "inactive" DR5 (Fig. 8a). We obtained similar results using agonistic, DR4- and DR5-specific antibodies. Thus, treatment of cells with UO126 led to enhancement of DR4-, but not DR5-mediated cell death, whereas Goe6983 was able to enhance cell death induced by both, mapatumumab and lexatumumab (Fig. 8b). Of note, it was not possible to quantify the influence of these inhibitors on TRAIL-mediated IL-8 induction since UO126 as well as oe6983 alone already dramatically reduced basal IL-8 secretion.

Thus, although the mechanism(s) of DR5 silencing for TRAIL in pancreatic tumor cells remain still unknown, pharmacological inhibitor(s) may provide a possibility to re-activate DR5 for TRAIL-induced death signaling.

Discussion

TRAIL induces cell death via activation of the two death receptors DR4 and DR5, two very similar proteins with an

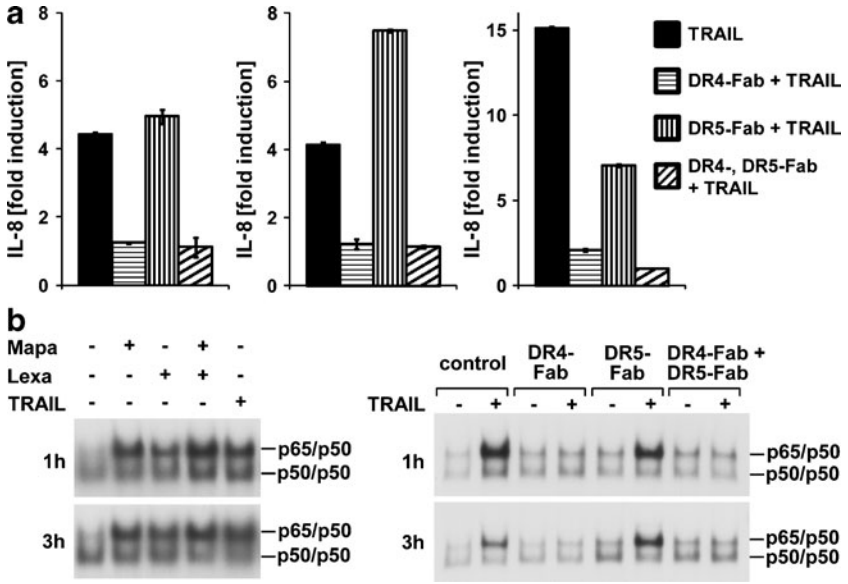

Fig. 7 TRAIL induces pro-inflammatory responses predominantly via DR4 in PDAC cells although both TRAIL receptors are functional. **a** TRAIL induces IL-8 predominantly via DR4. Cells (Colo357-left panel; Panc89-middle panel and PT45-right panel) were treated for 16 h with TRAIL (100 ng/ml) with or without blockade of DR4 (DR4-Fab) or DR5 (DR5-Fab) or both (DR4-Fab+DR5-Fab). IL-8 levels in cell culture supernatants were quantified by IL-8- ELISA. Data shown are representative results from at least three independent experiments performed in triplicates. **b** Analyses of TRAIL/antibody-induced NF–κB activity in Panc89 cells. *Left panel* shows that TRAIL as well as agonistic antibodies induce NF–κB activation. Cells were treated for 1 h or 3 h with TRAIL (100 ng/ml), mapatumumab (10 μg/ml), or lexatumumab (10 μg/ml). NF–κB activities were determined in nuclear extracts by EMSA. *Right panel* shows that TRAIL induces NF–κB exclusively via DR4. Cells were treated with TRAIL (100 ng/ml) with or without specific blocking of either DR4 (DR4-Fab) or DR5 (DR5-Fab) or both. Following stimulation nuclear extracts were prepared and analyzed for NF–κB activity by EMSA

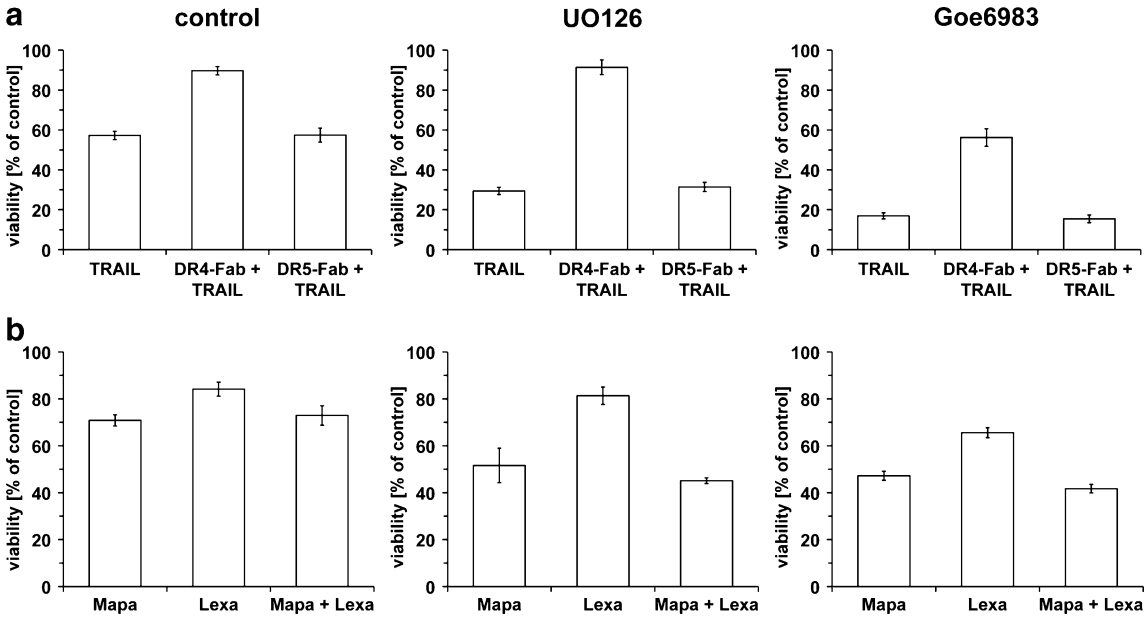

Fig. 8 Influence of MEK- and PKC-inhibition on TRAIL-receptor-mediated cell death in Panc89 cells. **a** Panc89 cells were pre-treated with the MEK-inhibitor UO126 (10 μM) or the PKC inhibitor Goe6983 (20 μM) 30 min prior to treatment with TRAIL (100 ng/ml) for 16 h. For inhibition of DR4 or DR5, corresponding Fab fragments were added to the cell culture medium. Viability was determined by crystal violet staining. Data represent means±SD (n=4). **b** Panc89 cells were pre-treated with MEK-inhibitor UO126 (10 μM) or Goe6983 (20 μM) 30 min prior to treatment (16 h) with mapatumumab (10 μg/ml), lexatumumab (10 μg/ml), or mapatumumab+lexatumumab (10 μg/ml each). Viability of cells was measured by crystal violet staining. Data represent means±SD (n=4)

amino acid sequence homology of 58% [5]. Both receptors are also able to induce non-apoptotic signaling, and so far, it is not known if they have any unique function [7]. Likewise, little is known about the usage of the two TRAIL death receptors in particular cell types. Recently, by using receptor-specific TRAIL mutants, Kelley et al. showed that tumor cells of different origin preferentially use DR5 to signal cell death [28]. Using other tools, namely agonistic antibodies, MacFarlane et al. showed that chronic lymphocytic leukemia cells induce apoptosis via DR4 [29]. To the best of our knowledge, there are no reports analyzing in detail side by side the effects of TRAIL-treatments with respect to the particular receptor usage and agonistic antibodies in the same type of tumor cells.

In the present study, we therefore analyzed apoptotic and non-apoptotic responses to TRAIL as well as to the agonistic DR4- and DR5-specific monoclonal antibodies mapatumumab and lexatumumab. In PDAC cells, representing a potential target of TRAIL-based therapies, we unraveled substantial differences in the killing activity of TRAIL and agonistic antibodies. We show that, although both death receptors are expressed at the cell surface of PDAC cells, TRAIL induces apoptosis and IL-8 secretion predominantly via DR4. The inability of TRAIL preparation used in our study to stimulate DR5-dependent signaling could also not be explained by insufficient ligand oligomerization, as it has been described for some forms of TRAIL [30] since Jurkat cells, expressing only DR5 at the cell surface, were efficiently killed. Interestingly, direct triggering of either DR4 or DR5 with mapatumumab or lexatumumab, showed that both receptors are capable to induce apoptosis and IL-8 secretion. Conforming to these data, DISC analysis performed after TRAIL stimulation revealed that caspase-8 is only cleaved in association with DR4 but not in the DR5 complex. Stimulation of cells with mapatumumab or lexatumumab, however, resulted in DISC formation and association with cleaved caspase-8. Furthermore, non-apoptotic TRAIL signaling was also mediated predominantly by DR4, but both receptors were able to activate NF–κB and to induce IL-8 secretion when stimulated by mapatumumab- or lexatumumab antibodies.

These results suggest that there exist differences between the mechanisms by which TRAIL and agonistic antibodies activate DR5. Indeed, we found that TRAIL induces, although not very efficiently, the formation of heteromeric DR4/DR5 complexes, while the agonistic antibodies exclusively induced the formation of a receptor-homotypic DISC. However, whether this is causally linked to the differential responsiveness of DR5 to TRAIL and lexatumumab is unclear and needs further investigations.

Although we observed the formation of heterocomplexes of DR4 and DR5 after stimulation with TRAIL, we did not find any cleaved caspase-8 in complexes isolated by immunoprecipitation of DR5. Furthermore, inhibition of DR5 did not rescue TRAIL-mediated cell death. It is therefore possible that at least in PDAC cells, DR4/DR5 heterocomplexes are not functional. Recently, it has been shown that DcR2/TRAIL-R4 can form complexes with DR5 thereby preventing the formation of functional DISC [31]. Although Panc89 cells, which we used for most of our studies, express DcR2 at the cell surface, we did not find any association of DR5 with DcR2 in immunoprecipitation experiments done either prior or following TRAIL treatment (data not shown). Obviously, this mechanism is not responsible for the "silencing" of DR5 for TRAIL-mediated signaling in PDAC cells. Interestingly, we found that PDAC cells, especially Panc89 cells, expressed additionally to the approximately 48 and 40 kDa forms of DR5, a faster migrating variant of DR5 of about 32 kDa. Recently, Wagner et al. identified a non-glycosylated form of DR5, which has a similar size [32]. Furthermore, the authors showed that *O*-glycosylation of DR4 and DR5 promote death receptor clustering following ligand binding and enhances apoptosis. Conversely, the non-glycosylated form efficiently inhibited DISC formation and consequently, apoptosis [32]. Importantly, we found that the 32 kDa form of DR5 was recruited to the TRAIL-induced DISC but was completely absent in lexatumumab-DISC. It is therefore likely that recruitment of the non-glycosylated DR5-form to the TRAIL-DISC accounts for DR5 silencing in TRAIL-mediated apoptosis in PDAC cells. Whether glycosylation of the small form of DR5 could re-activate DR5 for TRAIL signaling in PDAC cells or whether non-glycosylated DR5 and glycosylated form could form inactive heteromers remains to be shown.

By means of pharmacological inhibitors, we unraveled further differences between DR4- and DR5-induced responses. We found that inhibition of either PKC-or MEK-enhanced TRAIL-mediated cell death, but DR4- and DR5-mediated responses were differentially affected by these inhibitors. MEK inhibition potentiated DR4-mediated cell death, irrespective of whether cells were treated with TRAIL or with mapatumumab. In contrast, DR5-mediated response was not affected by this inhibitor. Interestingly, the PKC inhibitor Goe6983 enhanced apoptosis induction by both receptors, when signaling induced by agonistic antibodies was studied. Importantly, it was also able to "re-activate" DR5 for TRAIL-mediated cell death. Previously, we have shown that PKC and MEK are activated by TRAIL in PDAC cells [15, 18]. The important protecting role of PKC has also been demonstrated elsewhere [33–35]. In our present report, we showed that DR4- and DR5-mediated cell death is differentially affected by PKC- and MEK-inhibitor in Panc89 cells. Although the detailed mechanism of Goe6983-induced re-activation of DR5 for TRAIL-mediated cell death remains to be elucidated, it may

gain importance in clinical terms perspectives. We are currently trying to ascertain further differences in DR4- and DR5-mediated signaling as well as the mechanisms underlying Goe6983 sensitizing properties.

In conclusion, the results of this study unraveled signaling differences between the natural ligand TRAIL and specifically designed agonistic anti-DR4 and DR5 antibodies. We showed that TRAIL signals predominantly via DR4 although both receptors can be efficiently activated by respective agonistic antibodies. Proteomic-based analyses reveal DR4 and DR5 homocomplexes under these conditions, whereas TRAIL-induced hetero- complexes of DR4 and DR5. Of note, DR5 can be re-activated for TRAIL-mediated death signaling by pre-treatment of cells with PKC inhibitor Goe6983 offering a novel chance for clinical translation.

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