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ORIGINAL PAPER

TRAIL promotes membrane blebbing, detachment and migration of cells displaying a dysfunctional intrinsic pathway of apoptosis

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Abstract Recently, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL/Apo2L) has been shown to be a potential candidate for cancer therapy. TRAIL induces apoptosis in various cancer cells but not in normal tissues. Here we show that HCT116 and SW480 cells with a deficient mitochondrial apoptotic pathway were resistant to TRAIL-induced apoptosis, whereas HCT116 and SW480 cells with a functional mitochondrial apoptotic pathway underwent apoptosis upon exposure to TRAIL. Surprisingly, TRAIL induced phenotypic changes in cells with a dysfunctional mitochondrial apoptotic pathway, including membrane blebbing and a transient loss of adhesion properties to the substratum. Accordingly, TRAIL stimulated the ability of these cells to migrate. This behavior was the consequence of a transient TRAIL-induced ROCK1 cleavage. In addition, we report that Bax-deficient HCT116 cells exposed to TRAIL for a prolonged period lost their sensitivity to TRAIL as a result of downregulation of TRAIL receptor expression, and became resistant to combination of TRAIL and other drugs such as MG-132 and bortezomib. These findings may have important consequences for TRAIL anti-cancer therapy.

Keywords TRAIL · Membrane blebbing · ROCK1 · HCT116 Bax−/− · Cancer cell migration · Drug resistance · Bortezomib · Proteasome

Abbreviations

TRAIL	Tumor necrosis factor (TNF)-related apoptosis-inducing ligand
TNFR1	Tumor necrosis factor receptor-1
DISC	Death inducing signaling complex
TRAIL-R1	TRAIL receptor-1
TRAIL-R2	TRAIL receptor-2
MLC	Myosin light chain
ROCK1	Rho-associated, coiled-coil containing protein kinase 1
IAPs	Inhibitors of apoptosis
XIAP	X-linked inhibitor of apoptosis

Introduction

Tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) is a type 2 transmembrane protein whose extracellular domain shows homology with the extracellular domain of Fas ligand and TNF-α [1, 2]. TRAIL is

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normally expressed in the immune system and plays a critical role in antitumor immunity. TRAIL binds its receptors (DR4, also called TRAIL-R1 or DR5, also called TRAIL-R2), and triggers apoptosis via the extrinsic pathway. TRAIL receptors belong to the family of death receptors, which includes TNFR1 and Fas (Apo1, CD95) receptors [3–7]. These receptors display a death domain in their intracellular region. When bound to their ligands, they oligomerize and recruit the cytosolic protein FADD, which in turn recruits and activates caspase-8 [8, 9]. This expanding complex is called the death inducing signaling complex (DISC) [10]. Caspase-8 in turn cleaves and activates executioner caspases leading to apoptosis. In many cell types, the extrinsic pathway is linked to the intrinsic mitochondrial pathway by tBid. tBid, which is generated by the cleavage of Bid by caspase-8, activates apoptosis by recruiting Bax in the mitochondrial outer membrane [11]. This leads to permeabilization of the outer mitochondrial membrane, cytochrome c release, caspase-9 activation and ultimately to activation of executioner caspases and cell death.

Although TRAIL preferentially induces apoptosis in cancer cells, it also engages non-apoptotic signalling pathways leading to activation of pro-survival molecules, such as protein kinase C, phosphatidyl inositol 3 kinase (PI3K), Akt, nuclear factor kappaB (NF-kappaB) and mitogen-activated protein kinases [12–19]. These pathways stimulate transcription of genes encoding anti-apoptotic, angiogenic, mitogenic and cell migration-stimulating factors, raising the possibility of unwanted effects of TRAIL in cancer therapy.

Resistance of cancers to TRAIL is one of the major obstacles in TRAIL therapy. There are several causes for TRAIL resistance including the loss of expression of TRAIL-R1 and TRAIL-R2 on the plasma membrane, increased level of TRAIL-R3, TRAIL-R4, c-FLIP, Bcl-2, Bcl-xL, and loss of caspase-8 and -10 due to mutations [20–22]. Members of the inhibitors of apoptosis (IAPs) are reported to block the activity of caspase-9, -3 and -7 and high expression of XIAP, one of the IAP family members, has been shown to contribute to TRAIL resistance in a number of tumor cell lines [23]. While exploring the mechanisms of Bax activation induced by TRAIL, we observed an unexpected behavior of Bax−/− HCT116 human colon cancer cells. Upon TRAIL treatment, Bax−/− cells displayed robust membrane blebbing, and detached from the culture dish similar to Bax−/+ cells, however, the Bax−/− cells re-adhered to the culture dish after several hours and continued to proliferate. We have found that blebbing of Bax−/− cells occurs through caspase-3 mediated cleavage of ROCK1, a kinase important in cell membrane blebbing and motility. In vitro, TRAIL enhanced migration of Bax−/− cells. Moreover, a 24 h exposure of HCT116 Bax−/− cells to TRAIL conferred resistance to drug-mediated (such as MG-132 and bortezomib) sensitization of these cells to TRAIL-induced apoptosis. Cell detachment from the primary tumor is the first of a series of events required for metastasis. Our results raise the possibility that, in vivo, TRAIL anti-cancer therapy may facilitate migration of tumor cells in which the intrinsic mitochondrial pathway of apoptosis is impaired.

Materials and methods

Materials

Dulbecco's Modified Eagle Medium (DMEM), L-glutamine, fetal bovine serum (FBS), cell culture trypsin, MG-132 and Y-27632 were from Sigma; cell culture plates were from Nunc; Hoechst and Lipofectamine reagent were from Life Technologies; Bortezomib was from ChemieTek (USA); Hybond-P membranes were from GE Healthcare Life sciences; Annexin V-FITC was from BD Biosciences; The following antibodies were used. Bax (Neomarkers, clone 2D2), Bcl-xL (Santa Cruz, S-18), cleaved caspase-3 and GAPDH (Cell Signaling), caspase-3 (Alexis), caspase-8 (BD Pharmingen), PARP, ROCK1 (N-terminal), caspase-7 and XIAP (BD Transduction Laboratories), actin and ROCK1 [(C-terminal) (Sigma)], TRAIL-R1 and TRAIL-R2 (Exbio) and ppMLC (Generously provided by James Staddon, Eisai London Research Laboratories Ltd., London, UK).

Cell culture, transfections, and quantification of blebbing and detachment

Cell lines were cultured in DMEM with 10 % FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM glutamine, and maintained in 5 % CO_2 at 37 °C. HCT116 human colon cancer cell lines (HCT116 Bax−/+ and Bax−/−) that differ in the presence or absence of Bax were kindly provided by Bert Vogelstein, Howard Hughes Medical Institute. These isogenic cell lines were originally generated from HCT116 colon cancer cells [24]. HCT116 Bax−/+ cell lines over expressing Bcl-2 or Bcl-xL were generated by retroviral transduction of HCT116 Bax−/+ cells followed by cell sorting using Coulter EPICS Elite ESP, Beckman-Coulter-France, Villepinte, France [22].

In order to show that cleavage of ROCK1 is sufficient to induce membrane blebbing, a plasmid encoding an active form of ROCK1 [(ROCK1 (G1114opa); N-terminal domain of ROCK1 cleaved by caspase-3; the cDNA was kindly provided by Michael F. Olson, Beatson Institute for Cancer Research, UK] was used to transiently transfect HCT116 Bax−/− cells. TRAIL resistant SW480 cells (ATCC) were generated by transient transfection using a plasmid

encoding anti-apoptotic protein Bcl-xL. For the downregulation of caspase-3, HCT116 Bax−/+ and Bax−/− cells were transfected with siRNA targeting caspase-3 [Mission siRNA (ranking 1), SASI_Hs01_00139105] or universal negative control (SIC001) (Sigma) using Microporator MP100 (Digital Bio).

For blebbing and detachment analyses, cells were treated with 100 ng/ml of TRAIL or TRAIL (100 ng/ml) and Y-27632 (10 μM) for 5 h to allow membrane blebbing and detachment. Cells were then fixed with paraformaldehyde, stained with Hoechst and observed using phase contrast and fluorescence microscopy with a 63× objective (Zeiss Axiovert 135). The morphology of blebbing cells could easily be distinguished from that of non-blebbing adherent cells. The blebbing cells are also distinct from refringent, round cells that are undergoing mitosis. In order to compare dead and living cells, HCT116 Bax−/+ and Bax−/− cells were left untreated or treated with 100 ng/ml of TRAIL for 5 h. The cells were then stained with 5 μl/ml of Annexin V-FITC and 1 mg/ml of Hoechst in 1× Binding Buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) for 5–10 min in the dark and imaged. The Hoechst and annexin V stainings allowed us to differentiate between blebbing cells undergoing apoptosis (they displayed a fragmented and/or condensed nucleus, and were annexin V positive) and blebbing cells, which were resistant to apoptosis (their nucleus was intact and these cells were annexin V negative).

Combined TRAIL and proteasome inhibitor treatments, and apoptosis quantification

HCT116 Bax−/− cells were categorized into the following three groups: cells that had never been exposed to TRAIL (naïve cells); cells that had been pretreated with TRAIL for 24 h (pretreated cells); cells that received TRAIL for 24 h, then were left untreated for 48 h before a second exposure to TRAIL (sequentially treated cells). Each group of cells was left untreated or treated with TRAIL (100 ng/ml), MG-132 (10 μM), bortezomib (25 nM), TRAIL (100 ng/ml) and MG-132 (10 μM) or TRAIL (100 ng/ml) and bortezomib (25 nM). Cells were harvested after 5 h of treatment and apoptosis was detected using Annexin V-FITC as described earlier [25].

Time-lapse microscopy

HCT116 Bax−/− and Bax−/+ cells were plated in 35 mm glass bottom dishes (WillCo-dish, type 3522, WillCo Wells BV). The cultures were placed in a 37 °C chamber equilibrated with humidified air containing 5 % CO_2 while working with video microscopy. At 5 min before observation, the media was changed to fresh DMEM containing 10 % serum added with TRAIL (100 ng/ml). Time-lapse microscopy was performed with a Leica AF6000LX microscope using a 40× objective. The cells were imaged using differential interference contrast with a classical halogen lamp as the illumination source. Images were captured every 7 min and the movies were made out of the time-lapse series using ImageJ software.

Western blotting

Cells were resuspended in lysis buffer containing 10 mM HEPES, 300 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 % Triton X-100 (v/v), 0.1 % (w/v) sodium dodecyl sulphate (SDS), pH 7.4, and supplemented with 1× protease inhibitors (Roche Diagnostics, Germany). The lysate was centrifuged at 2,000×*g*, and the protein concentration was determined by Bradford assay (Bio-Rad). Equal amounts of protein were subjected to SDS–polyacrylamide gel electrophoresis, and transferred to Hybond-P membranes. Membranes were blocked with 5 % milk in PBST (PBS + 0.05 % Tween 20) and incubated overnight at 4 °C with primary antibodies. Blots were then washed in PBST and incubated for 1 h with secondary polyclonal antibodies coupled to horseradish peroxidase. Membranes were then washed in PBST and developed using chemiluminescence.

Immunocytochemistry and FACS analysis

To visualize the phosphorylation of the myosin light chain (MLC), HCT116 Bax−/− cells grown on coverslips were incubated in growth medium, with or without TRAIL for 5 and 24 h. The cells were then fixed in 4 % paraformaldehyde, permeabilized and immunostained with an antibody directed against ppMLC. For the quantification of TRAIL receptors, Bax−/− cells subjected to different treatments were stained with anti-TRAIL-R1 and anti-TRAIL-R2 antibodies followed by staining with FITC-conjugated secondary antibodies and analyzed by FACS.

Cell migration assay

Cell migration was quantified using 24 well cell migration assay kit (Trevigen) utilizing a modified Boyden chamber design with polyethylene terephthalate membrane. HCT116 Bax−/− and Bax−/+ cells were serum starved overnight. The following day, cells were re-suspended in serum free DMEM without or with TRAIL (100 ng/ml), Y-27632 (10 μM) or TRAIL (100 ng/ml) and Y-27632 (10 μM), and were added into the top inserts of the chamber. DMEM supplemented with 10 % serum was added into bottom wells of the chamber followed by incubation for 24 h. The cells that had migrated were quantified using Calcein-AM as described by the supplier (Trevigen).

Results

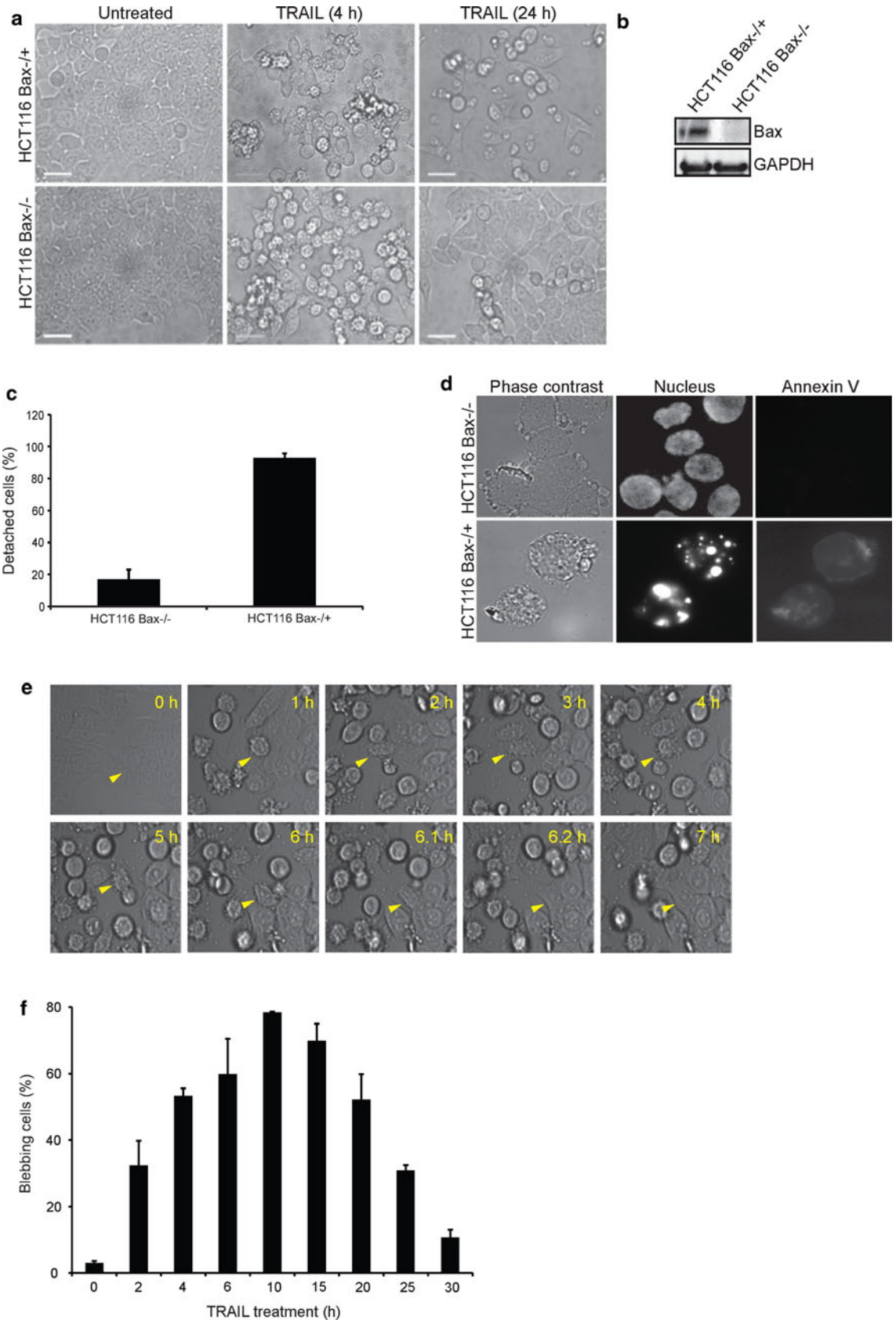
TRAIL promotes reversible membrane blebbing and detachment of Bax−/− HTC116 cells and of Bcl-xL overexpressing SW480 cells

We have compared the effect of TRAIL on Bax−/+ and Bax−/− HCT116 colon cancer cells early after addition of TRAIL. We observed that ∼100 % Bax−/+ cells displayed membrane blebbing and detached from the culture dish after addition of TRAIL (Fig. 1a, b, c and Movie 1). These cells were Annexin V positive and displayed a fragmented nucleus with condensed chromatin, two hallmarks of apoptosis (Fig. 1d). Bax−/− cells also displayed membrane blebbing (Fig. 1a, b, d), and ∼20 % cells were detached from the culture dish at 5 h TRAIL treatment (Fig. 1c). To our surprise, however, these blebbing cells remained Annexin V negative and their nucleus was intact (Fig. 1d). The Bax−/− cells started blebbing and detaching 1–2 h after TRAIL treatment, with a maximum of cells blebbing after ∼10 h (Fig. 1e, f and Movie 2). Thereafter, blebs began to disappear and the cells were able to re-attach within the 24 h following TRAIL treatment. Similar findings were obtained with HCT116 Bax−/+ cells overexpressing Bcl-2 or Bcl-xL (see Supplementary Fig. 1a, b). Moreover, different colon cancer cells, SW480 cells, overexpressing Bcl-xL, were found to resist to TRAIL-induced apoptosis (Supplementary Fig. 2a) and displayed a similar blebbing phenotype upon exposure to TRAIL (Supplementary Fig. 2b, c, d). Thus, at least two different cell types with a defect in the activation of the mitochondrial intrinsic pathway of apoptosis did not undergo apoptosis when exposed to TRAIL but showed membrane blebbing, and a transient loss of adhesion properties.

TRAIL promotes blebbing, detachment and migration of HCT116 Bax−/− cells by caspase-3 mediated cleavage of ROCK1

Whereas caspase-8 was equally activated in Bax−/+ and Bax−/− HCT116 cells upon TRAIL treatment (Fig. 2i), caspases-3 and -7 were not completely processed (p17 and p12 fragments were not detected) in the absence of Bax (Fig. 2a), which explained their resistance as previously reported [26, 33]. One of the caspase-3 substrates is ROCK1 that upon cleavage generates an active truncated kinase, which is responsible for membrane blebbing and cell detachment [27, 28]. We analyzed ROCK1 in Bax−/+ and Bax−/− cells and found that the protein was cleaved in both cell types upon TRAIL treatment (Fig. 2b). Interestingly, full length ROCK1 was re-expressed in Bax−/− cells that survived TRAIL treatment (Fig. 2b). In addition, by immunostaining, we observed a transient increase in the level of phosphorylation of MLC, a direct substrate of ROCK1, in TRAIL treated Bax−/− cells (Fig. 2c). MLC phosphorylation was undetectable in untreated cells or in TRAIL-treated cells that had re-attached (Fig. 2c). Importantly, in the presence of ROCK1 inhibitor Y-27632, membrane blebbing was efficiently blocked in HCT116 Bax−/− cells (Fig. 2d, e). In contrast, this treatment did not prevent detachment and death of Bax−/+ cells (Fig. 2d). Similar to HCT116 Bax−/− cells, blebbing and detachment of Bcl-xL overexpressing SW480 cells was significantly inhibited by the ROCK1 inhibitor Y-27632 (Supplementary Fig. 2b, c). On the other hand, whereas caspase-8, caspase-3, PARP and ROCK1 were efficiently cleaved in SW480 control cells, in Bcl-xL overexpressing SW480 cells, only caspase-8 and ROCK1 were efficiently processed while caspase-3 was partially processed (Supplementary Fig. 2e). These observations suggested the involvement of ROCK1 cleavage in the detachment of Bax−/− HCT116 and Bcl-xL overexpressing SW480 colon cancer cells. Since caspase-3 is known to be responsible for the cleavage of ROCK1 and cleaved ROCK1 is responsible for blebbing of cells [28, 29], we tested whether down-regulation of caspase-3 would have an impact on cell blebbing and detachment. We used RNA interference to down-regulate caspase-3 in HCT116 Bax−/− cells (Fig. 2f). As expected, the ROCK1 protein was processed less efficiently in caspase-3 siRNA transfected cells than in cells transfected with control siRNA (Fig. 2i). Consequently membrane blebbing was significantly reduced after TRAIL treatment (Fig. 2g, h). In addition, transient overexpression of a ROCK1 mutant with constitutive activity [28] (this mutant corresponds to the caspase 3-cleavage product of ROCK1), was sufficient to induce membrane blebbing (Supplementary Fig. 3a, b). Together, these data confirm that caspase-3 mediated cleavage of ROCK1 is essential and sufficient for the blebbing of Bax−/− cells.

Fig. 1 TRAIL treatment promotes reversible membrane blebbing in Bax−/− human colon cancer cells. **a** HCT116 Bax−/− and Bax−/+ cells were treated with TRAIL (100 ng/ml) and visualized by phase contrast microscopy. **b** Western blot showing the status of Bax in HCT116 Bax−/− and Bax−/+ cells. GAPDH is used as the loading control. **c** HCT116 Bax−/− and Bax−/+ cells were treated with 100 ng/ml of TRAIL for 5 h and the detached cells were collected, counted and expressed as percentage of the total number of cells in the well. Results are an average of three independent experiments. **d** HCT116 Bax−/− and Bax−/+ cells treated with TRAIL (100 ng/ml) for 5 h were stained with Hoechst and Annexin V-FITC. **e** Time-lapse images showing the changes in the morphology of HCT116 Bax−/− cells after treatment with TRAIL (100 ng/ml). Arrowhead tracks the morphology of a single cell, which starts to bleb after 1 h of TRAIL treatment and then gradually comes back to normal. **f** The number of blebbing cells were quantified from the time-lapse images taken at the indicated time points. The results are an average of three independent experiments


a
Untreated
TRAIL (4 h)
TRAIL (24 h)
HCT116 Bax-/+
HCT116 Bax-/-
b
HCT116 Bax-/+
HCT116 Bax-/-
Bax
GAPDH
c
Detached cells (%)
120
100
80
60
40
20
0
HCT116 Bax-/-
HCT116 Bax-/+
d
Phase contrast
Nucleus
Annexin V
HCT116 Bax-/-
HCT116 Bax-/+
e
0 h
1 h
2 h
3 h
4 h
5 h
6 h
6.1 h
6.2 h
7 h
f
Blebbing cells (%)
80
60
40
20
0
0
2
4
6
10
15
20
25
30
TRAIL treatment (h)

The blebbing phenotype shown by HCT116 Bax−/− cells upon TRAIL treatment led us to test whether this could enhance the migratory activity of these cells. Using Boyden chambers we compared migration of HCT116 Bax−/− and Bax−/+ cells. A significant increase in the migration of TRAIL treated HCT116 Bax−/− cells compared to untreated cells was observed (Fig. 3). Addition of the ROCK1 inhibitor Y-27632 suppressed migration of HCT116 Bax −/− cells in the presence of TRAIL (Fig. 3). As reported for other cell lines [30, 31] we found that treatment with Y-27632 alone was sufficient to prevent migration of HCT116 Bax−/+ and Bax−/− cells (Fig. 3) confirming ROCK1 as a potential target to inhibit cell migration.

High caspase-3 activation obtained by a combined treatment with TRAIL and proteasome inhibitor leads to apoptosis of HCT116 Bax−/− cells

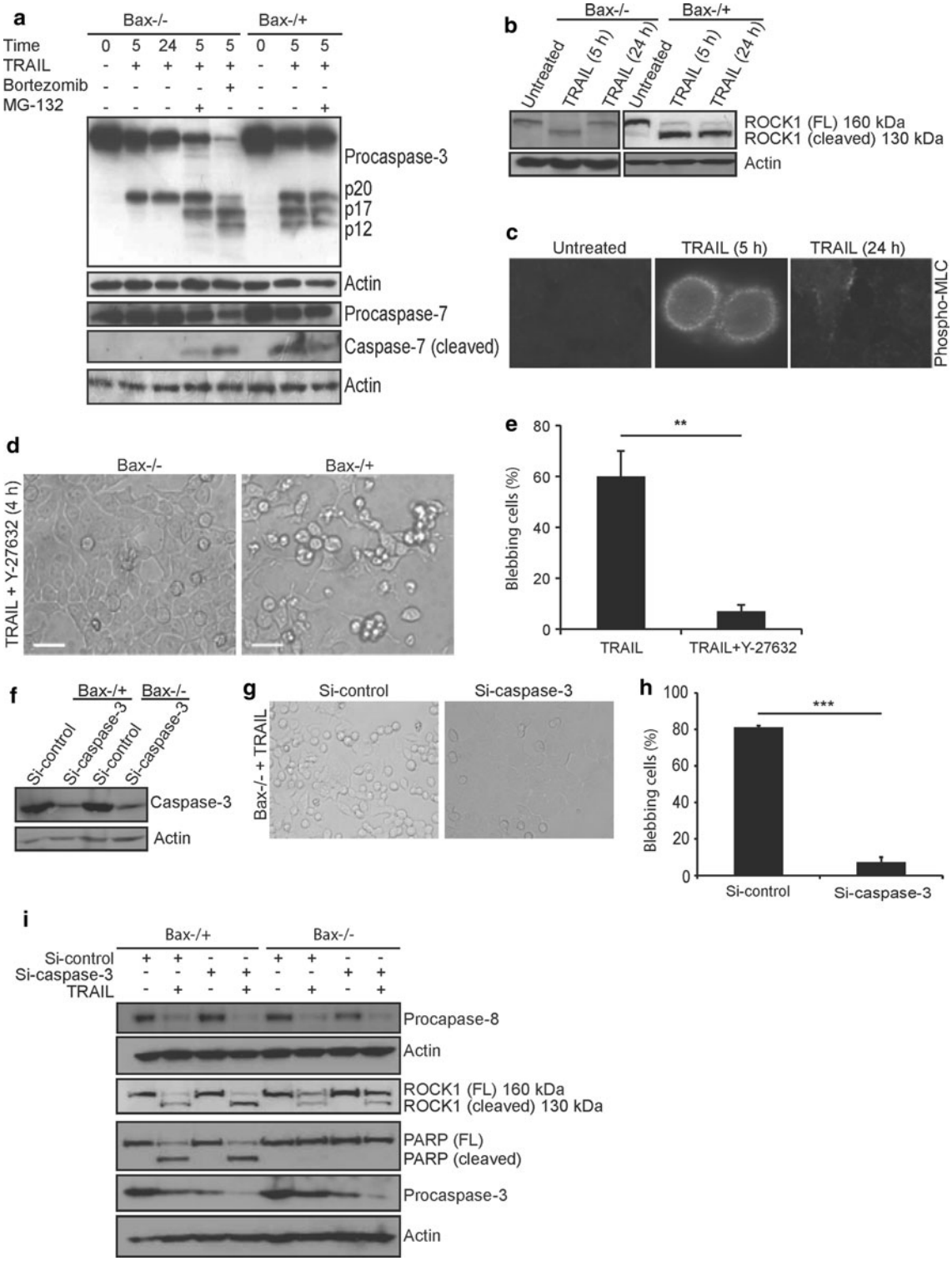
We found that activation of caspase-3 was responsible for the cleavage of ROCK1 leading to membrane blebbing and cell detachment. However, caspase-3 was unable to trigger apoptosis in Bax−/− HCT116 cells. Upon death receptor stimulation, caspase-3 follows a spatio-temporal activation, which initiates in the cytosol and leads to the translocation of at least some of the active fragments into the nucleus to cleave critical nuclear proteins [32, 33]. Immunostaining of Bax−/− HCT116 cells revealed that active caspase-3 was mainly localized at the cell periphery upon engagement of TRAIL receptor (Fig. 4a). We think that this subcellular localization may explain why only a limited number of substrates, including ROCK1, are cleaved by caspase 3 (Fig. 4a). However, this subcellular localization does not necessarily explain why caspase-3 is unable to undergo full activation and to trigger apoptosis in these cells.

Bax−/− HCT116 cells express high levels of IAPs such as XIAP [33], which are known to regulate caspase activity by ubiquitination and proteasome degradation. We reasoned that the small active fragments of caspase-3 (p17 and p12) produced by caspase-8, possibly at proximity of the plasma membrane, could be rapidly degraded by the proteasome before they reach their substrates in the nucleus and elsewhere. In order to test this hypothesis we inhibited the proteasome in TRAIL-treated cells using the chemical inhibitor MG-132. Proteasome inhibition has been previously reported to sensitize cells to TRAIL [34]. We found that Bax−/− cells treated with TRAIL and MG-132 underwent apoptosis, as did wild type cells in the presence of TRAIL alone (Figs. 4b, 2a, i). Similarly SW480 colon cancer cells overexpressing Bcl-xL were sensitized by the combined treatment of TRAIL and MG-132 (Supplementary Fig. 2a). Importantly, the use of MG-132 allowed detection of the short fragment of caspase-3 in HCT116 Bax−/− cells (Figs. 4c, 2a). To strengthen our interpretation that partial activation of caspase-3 is responsible for membrane blebbing and cell detachment, we downregulated caspase-3 in HCT116 Bax−/− cells with siRNA (Fig. 2f). Caspase-3-deficient cells did not detach and were found to be resistant to the combination of TRAIL and MG-132 (Figs. 2f, g, h, i, 4b, c).

Fig. 2 TRAIL promotes blebbing and detachment of HCT116 Bax−/− cells by caspase-3 mediated cleavage of ROCK1. **a** Activation of caspase-3 and -7 in HCT116 Bax−/− and Bax−/+ cells in response to TRAIL and proteasome inhibitors. Cells were left untreated or treated with TRAIL (100 ng/ml), TRAIL (100 ng/ml) and MG-132 (10 μM) or TRAIL (100 ng/ml) and Bortezomib (25 nM) for 5 h. Protein lysates prepared from these cells were separated by SDS-PAGE and analyzed for caspase-3 and -7 expression by Western blotting. Actin was used as the loading control. **b** Cell lysates were prepared from HCT116 Bax−/− and Bax−/+ cells left untreated or treated with TRAIL for 5 or 24 h and analyzed for ROCK1 by Western blotting. The blot is representative of three independent experiments. **c** Phosphorylation of MLC in untreated or TRAIL treated HCT116 Bax−/− cells for 5 and 24 h. **d** Effect of ROCK1 inhibitor Y-27632 (10 μM) on TRAIL-induced membrane blebbing in HCT116 Bax−/+ and Bax−/− cells. **e** Quantification of TRAIL-mediated membrane blebbing in HCT116 Bax−/− cells in presence and absence of Y-27632. Mean values ± SEM are shown for three independent experiments with $^{**}p < 0.01$. **f** Down regulation of caspase-3 by siRNA. HCT116 Bax−/− and Bax−/+ cells were microporated with siRNA for caspase-3 or with a control siRNA. The cells were harvested after 72 h and the cell lysates were analyzed for caspase-3 by Western blotting. Caspase-3 deficient HCT116 Bax−/− cells were treated with TRAIL (100 ng/ml) and imaged by phase contrast microscopy (**g**), and the number of blebbing cells was quantified (**h**). Mean values ± SEM are shown for three independent experiments with $^{***}p < 0.001$. **i** Downregulation of caspase-3 reduces the processing of ROCK1 in HCT116 Bax−/− cells. HCT116 Bax−/− and Bax−/+cells transfected with siRNA targeting caspase-3 or control siRNA were treated with or without TRAIL, and protein lysates were analyzed for ROCK1, caspase-3, caspase-8, PARP and actin by Western blotting

Acquired resistance of HCT116 Bax−/− cells to TRAIL

There are numerous reports showing that combining proteasome inhibition with TRAIL is a promising approach for the treatment of TRAIL resistant cancers [34–39]. Accordingly, we found that cells that had never been exposed to TRAIL (naïve cells) underwent apoptosis after exposure to TRAIL and a proteasome inhibitor (Fig. 5a, b). Interestingly, we observed that Bax−/− cells treated with TRAIL for 24 h (pretreated cells) became insensitive to a further co-treatment with TRAIL and proteasome inhibitors MG-132 or bortezomib (Fig. 5a, b). However, suspension of the TRAIL treatment for 48 h (sequentially treated cells) resensitized cells to the combined treatment of TRAIL and MG-132 or bortezomib (Fig. 5a, b). Moreover, we found that downregulation of caspase-3 rendered naïve Bax−/− cells insensitive to TRAIL and MG-132



co-treatment showing that these drugs work through activation of caspase-3 (Fig. 4b, c). To understand the mechanisms by which pretreated cells exposed to TRAIL acquired resistance to the combination of TRAIL and a proteasome inhibitor, we compared the status of caspase-3, caspase-8, XIAP and the expression of the TRAIL-R1 and TRAIL-R2 receptors in these cells. We observed that caspase-8 was significantly less processed in TRAIL-pretreated cells (Fig. 6a). Moreover, the amount of XIAP protein remained constant in TRAIL-pretreated cells,

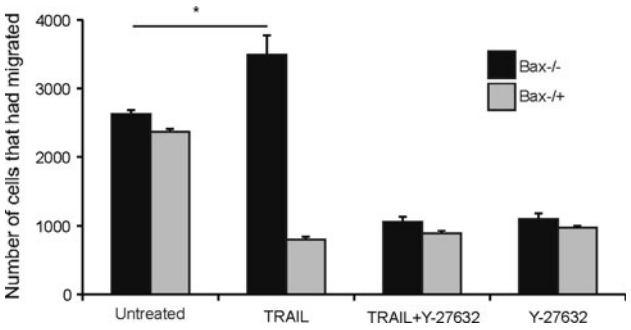


Fig. 3 TRAIL promotes in vitro migration of HCT116 Bax−/− cells. Migration of HCT116 Bax−/− and Bax−/+ cells in response to TRAIL (100 ng/ml), Y-27632 (10 μM) or TRAIL (100 ng/ml) and Y-27632 (10 μM) was analyzed using a modified Boyden chamber assay as described in the "Materials and methods" section. Mean values ± SD are shown for six independent experiments with $^{*}p < 0.05$

whereas it was decreased in naïve cells treated with TRAIL (Fig. 6a), possibly as a result of cleavage by caspase-3 [40]. Importantly, expression of both TRAIL-R1 and TRAIL-R2 receptors was also reduced in pretreated cells compared to naïve or sequentially treated cells (Fig. 6b). Interestingly, whereas the level of TRAIL-R2 returned to a normal value in sequentially treated cells, the level of TRAIL-R1 remained low (Fig. 6b). Together, these results suggest that the desensitization of Bax−/− cells to TRAIL could be in part due to decreased expression of TRAIL-R1 and TRAIL-R2 at the cell surface and that re-sensitization of these cells to TRAIL after interruption of the treatment may be due to TRAIL-R2 re-expression.

Discussion

In this paper we show that upon TRAIL treatment, two different cells lines, HCT116 and SW480 cells, lacking an efficient intrinsic pathway not only resist apoptosis, but also display membrane blebbing and an enhanced capability to migrate. Membrane blebbing and enhanced migratory activity of these cells lies in the cleavage of ROCK1 by caspase-3 since inhibition of ROCK1 inhibited both processes. TRAIL-induced membrane blebbing and

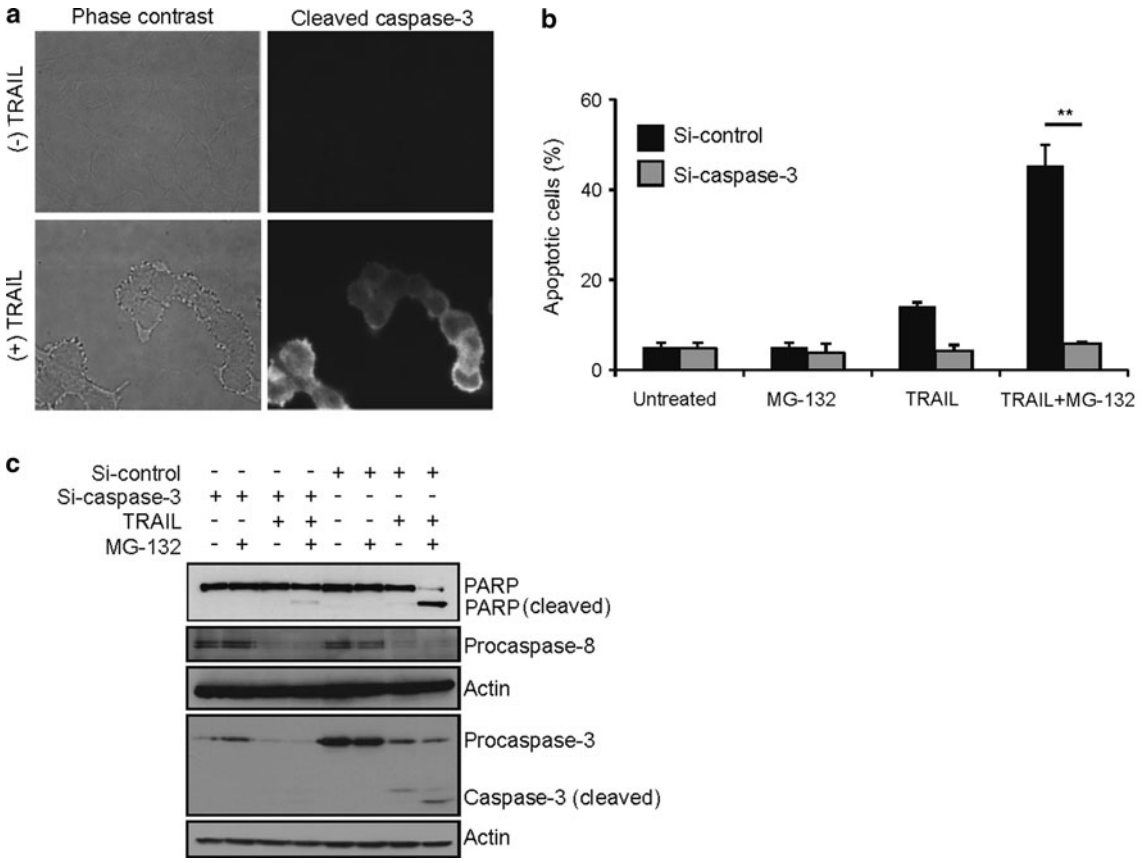


Fig. 4 Potentiation of TRAIL with a proteasome inhibitor. **a** Immunofluorescence analysis of active caspase-3 in untreated and TRAIL-treated HCT116 Bax−/− cells for 5 h. **b** Quantification of apoptosis using Annexin V staining of control or caspase-3-deficient HCT116 Bax−/− cells treated for 5 h with TRAIL (100 ng/ml) and the proteosome inhibitor MG-132 (10 μM). Mean values ± SEM are shown for four independent experiments with $^{**}p < 0.01$. **c** Cell lysates from these cells were analyzed for PARP, caspase-8, caspase-3 and actin by Western blotting

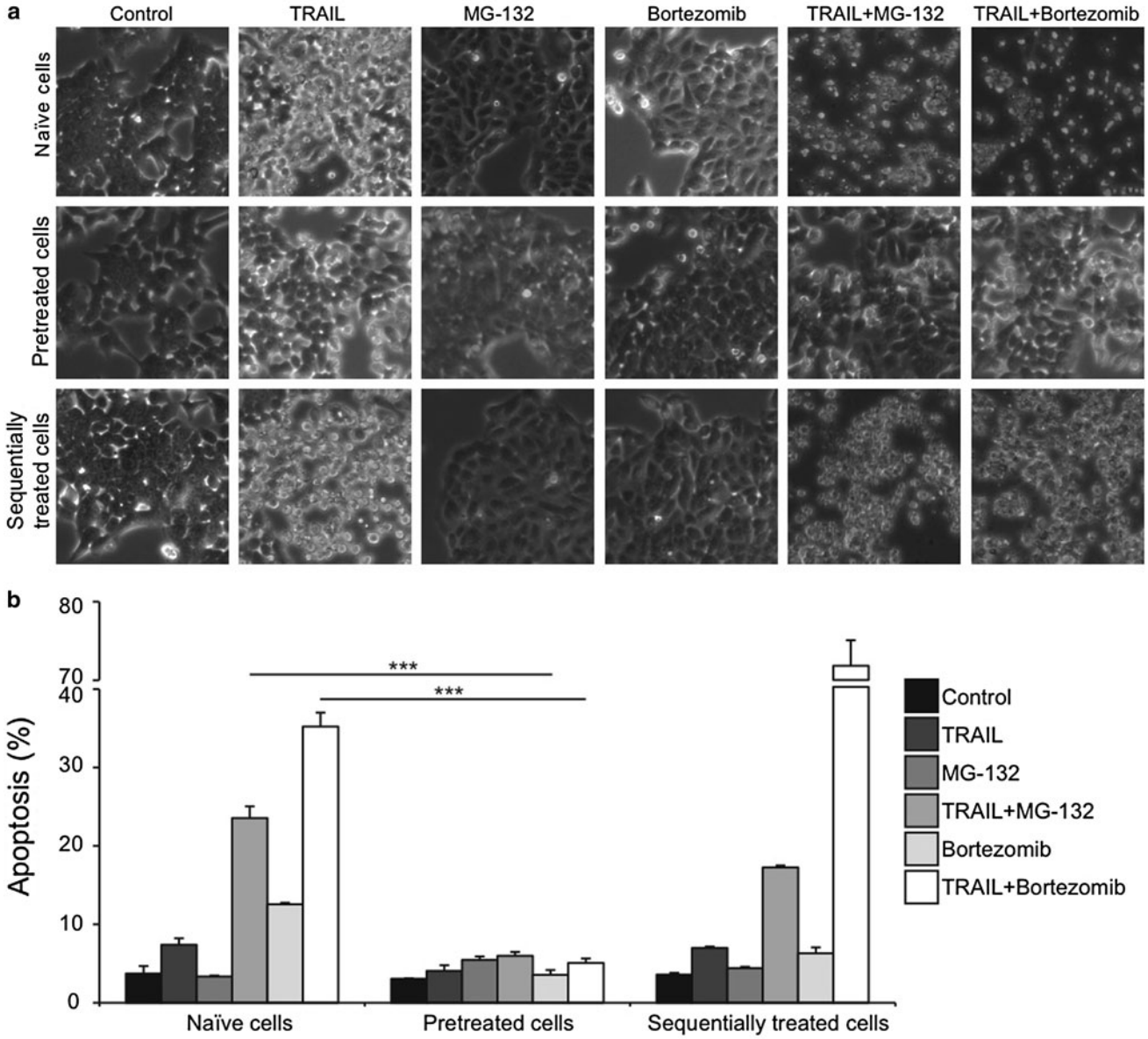


Fig. 5 Acquired resistance to TRAIL. **a** Naïve cells, pretreated cells and sequentially treated cells (see "Materials and methods" section) were treated with TRAIL (100 ng/ml), MG-132 (10 μM), Bortezomib (25 nM), TRAIL (100 ng/ml) and MG-132 (10 μM) or TRAIL (100 ng/ml) and bortezomib (25 nM) for 5 h, and imaged. **b** The treated cells were harvested and apoptosis was measured by Annexin V staining. Mean values ± SEM are shown for three independent experiments with $***p < 0.0001$

adoption of an amoeboid morphology has previously been described ([41, 42], A. Ashkenazi, personal communication). The process of membrane blebbing is not specific for apoptosis, as this has been observed under many physiological and pathological conditions such as embryonic development, cell division, cell spreading and metastasis [43, 44]. Many cancer cell lines such as M2 melanoma or migrating Walker carcinoma show membrane blebbing when they move [45]. During metastasis, cancer cells can either attain a mesenchymal mode of motility utilizing proteolytic mechanisms or an amoeboid blebbing mode of motility, or both to bypass tissue barriers before dissemination into lymphatic or blood vessels [46–48]. The fact that TRAIL promotes cell blebbing and migration is consistent with previous observations made in vitro [42, 49] and in vivo [50]. In the latter case, orthotopically transplanted human pancreatic ductal adenocarcinoma cells in the pancreas of severe combined immunodeficiency mice led to a dramatic increase in metastatic spread upon TRAIL treatment [50].

HCT116 cells are described as type II cells since they require the intrinsic pathway to activate executioner

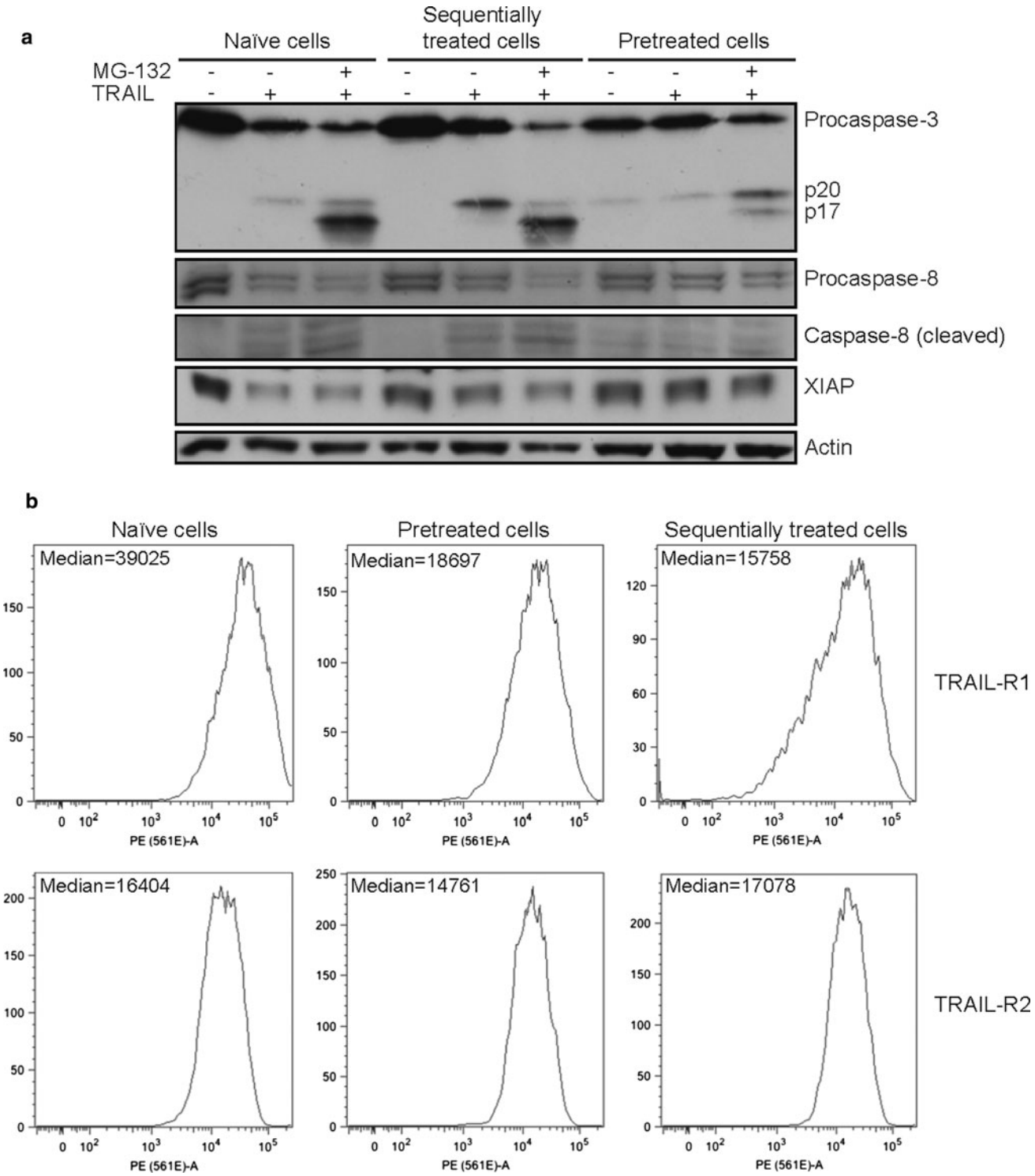


Fig. 6 TRAIL receptors are downregulated in pretreated HCT116 Bax−/− cells. **a** Cell lysates prepared from HCT116 Bax−/− cells at the indicated treatment conditions were analyzed for expression of caspase-3, caspase-8, XIAP and actin. **b** Analysis of cell surface expression of death receptors on HCT116 Bax−/− cells by flow cytometry. Cells as indicated in the treatment conditions were stained for anti-TRAIL-R1 or anti-TRAIL-R2 antibodies, counterstained with FITC conjugated secondary antibodies and analyzed by FACS

caspases and undergo apoptosis upon engagement of TRAIL receptors. Activation of the intrinsic pathway, which leads to the release of Smac/DIABLO from mitochondria, is required to counteract IAPs and to allow efficient caspase-3 activation. Accordingly, we observed that in the absence of a functional intrinsic pathway due to

the absence of Bax in HCT116 cells or due to the overexpression of Bcl-xL in SW480 cells, the level of active caspase-3 was significantly decreased after TRAIL treatment. This level was, however, sufficient to cleave ROCK1 but insufficient to cleave other key substrates and execute the cell. This result is in agreement with previous data showing that activation of caspase-3 is compartmentalized in many cells types [51, 52] and can be compatible with cell proliferation, survival and metastasis [53–59]. A wave of caspase-3 activation has also previously been reported to diffuse from underneath the plasma membrane to the nucleus [32, 60]. In Bax−/− HCT116 and Bcl-xL overexpressing SW480 cells this gradient of activation appears to be interrupted in the cytosol, due to the lack of Smac/DIABLO release and the persistence of a high IAP activity. Previous data have shown that an interaction between the cleaved form of caspase-3 (p24) and XIAP, one of the IAP family members, prevents the complete processing of caspase-3 in HCT116 Bax−/− cells [33]. Moreover, several of the human IAPs (XIAP, c-IAP1, and c-IAP2) are known for their ability to inhibit caspases directly [61, 62]. It was also reported that XIAP acts as a ubiquitin-protein ligase for caspase-3 [63]. Using constitutively active mutants of caspase-3, the authors have shown that XIAP promotes the degradation of the active-form of caspase-3 in a proteasome-dependent manner. In agreement with these studies, we found that inhibition of the proteasome, together with TRAIL, was accompanied by complete processing of caspase-3 and cell death. Thus inhibition of the proteasome transformed type II cells into type I cells.

Use of proteasome inhibitors has emerged as a promising approach for treating a number of cancers. Proteasome inhibitors enhance TRAIL-induced apoptosis in various tumor cells including colon cancer cells [35–39, 64]. Interestingly, we observed that a continuous treatment of Bax−/− cells with TRAIL rendered these cells resistant to the combination of TRAIL and a proteasome inhibitor. Previously published data have shown that acquired resistance to TRAIL could occur through c-Cbl-mediated downregulation of TRAIL receptors TRAIL-R1 and TRAIL-R2 [65]. In agreement with these findings, we found a decreased surface expression of TRAIL-R1 and TRAIL-R2 after exposure of Bax−/− HCT116 cells to TRAIL for 1 day. Removal of TRAIL from the culture medium for 2 days allowed these cells to recover expression of TRAIL-R2. We cannot exclude that in addition to the decreased surface expression of the receptors, other mechanisms may be at work to explain desensitization of the cells to TRAIL.

In conclusion, our observations, although limited to in vitro studies, are important to consider from a therapeutic point of view since they indicate that continuous TRAIL treatment can promote tumor resistance. Moreover, they point to the risk of inducing cell migration, and possibly metastasis, if TRAIL would be used alone to eradicate tumor cells displaying a deficient intrinsic mitochondrial pathway. In order to prevent cell detachment and migration, compounds such as ROCK1 inhibitors could be of great value. Finally our results raise the intriguing possibility that the immune system may be deleterious for the organism when fighting against tumors, since through expression of TRAIL or other death ligands of the TNF-α family, it may elicit tumor cell migration and thereby greatly influence disease outcome. All these hypotheses will have to be tested in vivo.

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