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TRAIL induces apoptosis but not necroptosis in colorectal and pancreatic cancer cells preferentially via the TRAIL-R2/DR5 receptor

Zuzana Nahacka[a,b], Jan Svadlenka[a,1], Martin Peterka[a,2], Marie Ksandrova[a], Simona Benesova[a,b], Jiri Neuzil[b,c], Ladislav Andera[a,b,*]

[a] Institute of Molecular Genetics, Czech Academy of Sciences, Prague, Czech Republic
[b] Institute of Biotechnology, Czech Academy of Sciences, Vestec, Czech Republic
[c] School of Medical Science, Griffith University, Southport, Qld, Australia

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a cytokine that can trigger apoptosis in many types of human cancer cells via engagement of its two pro-apoptotic receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5). TRAIL can also activate several other signaling pathways such as activation of stress kinases, canonical NF-κB signaling and necroptosis. Though both receptors are ubiquitously expressed, their relative participation in TRAIL-induced signaling is still largely unknown. To analyze TRAIL receptor-specific signaling, we prepared *Strep*-tagged, trimerized variants of recombinant human TRAIL with high affinity for either DR4 or DR5 receptor. Using these receptor-specific ligands, we examined the contribution of individual pro-apoptotic receptors to TRAIL-induced signaling pathways. We found that in TRAIL-resistant colorectal HT-29 cells but not in pancreatic PANC-1 cancer cells, DISC formation and initial caspase-8 processing proceeds comparably via both DR4- and DR5-activated signaling. TRAIL-induced apoptosis, enhanced by the inhibitor of the Bcl-2 family ABT-737, or by the translation inhibitor homoharringtonine, proceeded in both cell lines predominantly via the DR5 receptor. ShRNA-mediated downregulation of DR4 or DR5 receptors in HT-29 cells also pointed to a stronger contribution of DR5 in TRAIL-induced apoptosis. In contrast to apoptosis, necroptotic signaling was activated similarly by both DR4- or DR5-specific ligands. Activation of auxiliary signaling pathways involving NF-κB or stress kinases proceeded under apoptotic conditions mainly in a DR5-dependent manner, while these signaling pathways were during necroptosis similarly activated by either of these ligands. Our study provides the first systematic insight into DR4 − /DR5-specific signaling in colorectal and pancreatic cancer cells.

1. Introduction

TRAIL ligand (Apo-2L, TNFSF10), a member of the TNF ligand superfamily is expressed mainly by cells of the immune system. It can induce apoptosis in many types of human cancer cells [1–4]. However, depending on the tumor type, and the concurrent signaling, TRAIL-triggered cellular responses can also be pro-tumorigenic, enhancing cell survival and proliferation via non-canonical signaling [5–7]. Human TRAIL ligand binds two pro-apoptotic receptors; DR4 (TRAIL-R1) and DR5 (TRAIL-R2) and three decoy receptors DcR1 (TRAIL-R3), DcR2 (TRAIL-R4) and osteoprotegerin, which not only compete with DR4/5 for the ligand binding, but are also capable of transducing non-apoptotic pro-survival signaling as shown for the truncated DcR2. Binding of TRAIL to DR4 or DR5 triggers formation of the death-inducing signaling complex (DISC), containing the core adaptor protein FADD, pro-caspase-8 and the anti-apoptotic protein c-FLIP, which represents a platform for proximity-based, self-processing and activation of the initiator caspase-8, essential for TRAIL- or FasL-induced apoptotic signaling. In addition to pro-apoptotic signaling, TRAIL is capable of inducing a process called necroptosis in cells with inhibited caspases and free RIP1 kinase. Necroptosis proceeds by triggering the formation of the RIP1-activating intracellular complex, necrosome, containing FADD, c-FLIP, caspase-8, and RIP1 and RIP3 kinases [8]. Moreover, TRAIL can induce pro-survival NF-κB and MAP/stress kinase signaling similarly as FasL, involving the cytosolic complex consisting of FADD, TRADD, TRAF2, caspase-8, RIP1, NEMO and other proteins, (recently reviewed in [9]).

In 1999, Walczak and colleagues showed that recombinant human and murine TRAIL ligands can selectively and effectively trigger

* Corresponding author at: Institute of Biotechnology, Czech Academy of Sciences, Prumyslova 595, 252 50 Vestec, Czech Republic.
E-mail address: andera@ibt.cas.cz (L. Andera).
[1] Present address: Biorad CZ, Prague, Czech Republic.
[2] Present address: Institute of Biochemistry, ETH Zürich, Zürich, Switzerland.

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apoptosis of transformed cells also in vivo, and unlike other death-inducing ligands of the TNF family, systemic application of TRAIL did not manifest severe side effects, e.g. hepatotoxicity. This boosted interest in the TRAIL pro-apoptotic signaling and led to the development of a number of recombinant ligands and agonistic anti-DR4/−5 antibodies. However, clinical trials using recombinant TRAIL Dulanermin (Genentech) and several humanized agonistic antibodies were rather disappointing, having shown only a limited anti-tumor effect [10]. The mechanism underlying the resistance of cancer cells to TRAIL-induced death could be a result of a variety of factors acting at different levels of the TRAIL signaling pathway, such as suppressed cell surface expression of its pro-apoptotic receptors, impaired activation or activity of caspase-8 due to mutations, epigenetic silencing or ubiquitination, or also overexpression of a number of anti-apoptotic proteins such as the inhibitor of caspase-8 c-FLIP, or inhibitors of apoptosis (IAP) family members and anti-apoptotic proteins from the Bcl-2 family [11]. Moreover, recent publications document that tumor cells can, under suppressed apoptosis, hijack TRAIL-induced signaling for proliferation and secretion of pro-tumorigenic soluble factors [7,12–14].

The expression of two death-inducing TRAIL receptors in human and monkey cells compared to other mammals, which have only one pro-apoptotic TRAIL receptor and only 46–48% sequence identity of DR4 with DR5, suggests a structural, functional and regulatory difference between both receptors. Indeed, several studies with selective recombinant TRAIL ligands or DR4−/DR5-specific agonistic antibodies, have indicated possible selectivity of DR4 vs. DR5 apoptotic signaling in tumor cells of various origins. For example, pancreatic carcinoma, chronic lymphocytic leukemia or mantle cell lymphoma prefer DR4 for the induction of apoptosis; other types, such as colorectal or other epithelial cancer cells appear to prefer DR5 as an apoptosis inducer [15–20]. TRAIL receptor-specific ligands or agonistic antibodies have also increased affinity for their cognate receptor but very low affinity for decoy receptors, and thus might induce more robust and faster induction of cell death in tumor cells [21]. In our study, we aimed to analyze in detail the canonical and non-canonical signaling from our modified and enhanced TRAIL receptor-specific ligands in a model of colorectal and pancreatic cancer cells. We show that the DR5 receptor plays a major role in the induction of apoptosis as well as of non-canonical signaling in colorectal HT-29 cells, but not in the induction of TRAIL-triggered necroptosis. We also document preference for DR5 in apoptosis induction in the pancreatic cancer PANC-1 cells.

2. Materials and methods

2.1. Twin-Strep-tag TRAIL ligand variants and reagents

Twin-*strep*-tag in-frame with bacteriophage trimerization (TRI) motif [22] was synthetized (GeneScript) and sub-cloned upstream of the human TRAIL cDNA (extracellular part, amino acids 95–271) into pBKSII, resulting in pBKSII- TST-TRI-TRAIL-wt. TRAIL receptor-specific mutants DR4.02 (G131R/R149I/S159R/N199R/K201H/S215D, 4C7 mutant; [21]), DR5.01 (E195R/D269H; [19]) and DR5.02 (Y189N/R191K/E195R/H264R/I266L/D269H; [23]) were synthetized (Genscript) and sub-cloned into the pBSKII-TST-TRI plasmid. TST-tagged TRAIL ligands were inducibly expressed in the BL21-AI bacterial strain (Thermo Fisher), and proteins were purified using Gravity flow Strep-Tactin Sepharose column (IBA), according to the manufacturer's protocol.

Apoptosis was induced using human recombinant TRAIL ligand variants (see above) in combination with homoharringtonine (Sigma-Aldrich) or ABT-737 (Selleck Chemicals). TRAIL-mediated necroptosis was induced in cancer cells pre-treated with the IAP inhibitor birinapant (Selleck Chemicals) and pan-caspase inhibitor *Z*-VAD-FMK (Enzo LS). Necroptosis inhibitors necrostatin-1 and necrosulfonamide were obtained from Selleck Chemicals and from Tocris, respectively.

2.2. Cell lines and culture conditions

Human colorectal adenocarcinoma cell line HT-29, human pancreatic adenocarcinoma cell line PANC-1 and human kidney epithelial cells HEK293T were obtained from the ATCC. B-cell lymphoma cell line Ramos and the acute T-cell leukemia cell line Jurkat were kindly provided by V. Horejsi (Institute of Molecular Genetics, Czech Academy of Sciences) and were originally also obtained from ATCC.

The cells were cultured in DMEM (HEK 293T, HT-29, PANC-1) or in RPMI (Jurkat, Ramos) supplemented with v/v 10% FCS (Life Technologies), L-glutamine and antibiotics. In the case of PANC-1, non-essential amino acids (Life Technologies) were added to the medium. Cells were kept in a humidified incubator at 20% oxygen, 5% CO_2 and 37 °C. All unspecified media and cell culture additives were purchased from Sigma.

2.3. Preparation of recombinant lentiviruses and transduction of cells

Recombinant lentiviruses were obtained from calcium-phosphate transfected HEK 293T cells using packaging plasmids psPAX2 (Addgene, 12260) and pMD2.G (Addgene, 12259) together with either pLKO empty, pLKO non-targeting shRNA or pLKO shRNA against DR4 and DR5 receptors (shDR4_#1 TRCN0000005934, shDR4_#2 TRCN0000005935, shDR5_#1 TRCN0000005929, shDR5_#4 TRCN0000005932, shDR5_#5 TRCN0000005933). The medium containing lentiviral particles was harvested 36 to 48 h post-transfection, and the viral particles were precipitated using PEG-it (System Biosciences). Target cells were transduced with viruses at multiplicity of infection MOI 5–10 and selected for puromycine resistance (2 μg/ml, Invivogen).

2.4. Western blotting

The cells were lysed in the standard 2 × Laemmli buffer (50 mM Tris-HCl pH 6,8; 20% glycerol; 4% SDS; 0.02% bromophenol blue, 200 mM DTT). The samples were subjected to SDS-PAGE, proteins were transferred onto a nitrocellulose membrane (Hybond ECL; Amersham), and proteins visualized using individual primary antibodies (if not stated otherwise, primary antibodies were obtained from Cell Signaling): caspase-8 1C12 (9746); caspase-3 cleaved (9661); IκB-α (4814); phospho IκB-α (2859, Ser32); NF-κB p65 (4764); phospho NF-κB (3033, Ser536); phospho SAPK/JNK (9255S, Thr183/Tyr185); p38/MAPK (9212); phospho p38/MAPK (9211S, Thr180/Tyr182); phospho MLKL (14516, Thr357/Ser358); PARP (9532); caspase-3 (804-305-C100 Enzo); DR4 (8414, Abcam); MLKL (184718, Abcam); DR5 (D3938 Sigma-Aldrich); FADD (610400, BD); RIP1 (610458, BD); SAPK/JNK (sc-571, Santa Cruz); actin (1615, Santa Cruz); RIP3 (7604, MAB), in combination with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch). The protein-antibody complexes were visualized by Western Bright ECL (Advansta) or SuperSignal West Femto Maximum Sensitivity (Thermo Scientific).

2.5. Death inducing signaling complex (DISC) precipitation

Cells grown to approximately 80% confluence were rapidly cooled and then incubated on ice for 20 min (to prevent internalization of the receptors). Twin-*Strep*-tag labeled TRAIL ligand variants (TST-TRI-TRAIL) were added to the concentration of 1 μg/ml for 15 min, then the cold medium containing TRAIL was replaced with the 37 °C medium without TRAIL, and the cells were incubated at 37 °C. At selected time points, the cells were washed with cold PBS, the cell pellets were lysed in ice-cold lysis buffer (1% NP-40, 20 mM Tris–Cl pH 7.5, 150 mM NaCl, 10 mM EDTA, 10% glycerol) and supplemented with inhibitors of proteases (Complete, Roche) and phosphatases(10 mM NaF and 1 mM Na_3VO_4). Lysates were adjusted to the same protein concentration and the same amount of protein (2 mg) was incubated with Streptactin

beads (IBA) overnight at 4 °C and then washed 3–4 times with 1 volume of ice-cold lysis buffer; the bound proteins were directly eluted with 2 × Laemmli sample buffer (95 °C, 5 min) and analyzed by western blotting.

2.6. Flow cytometry analysis

For all flow cytometry assays, the cells were seeded in 12 or 24 well plates and harvested at 80% confluency. For annexin V-FITC assays, cells were harvested by trypsinization, washed with ice-cold PBS, then re-suspended in the annexin-binding buffer, and incubated on ice with annexin V-FITC (Apronex) at the final concentration of 2 μg/ml for 20 min. After adding Hoechst 33258 to the final concentration of 0.5 μg/ml, the samples were analyzed by using the LSRII flow cytometry (Beckton Dickenson).

For the analysis of death receptor expression, cells were harvested by mild trypsinization, then washed with ice-cold PBS, and non-specific interactions were blocked with PBS containing 0.2% gelatine and 0.1% sodium azide (PBS-GA). The samples were incubated on ice with the primary antibodies against DR4 (10-403, EXBIO) and DR5 (11-461, EXBIO) receptors at the final concentration of 10 μg/ml for 30 min. After incubation, cells were washed twice with ice-cold PBS-GA and incubated with the secondary goat anti-mouse IgG1 conjugated with phycoerythrin (final concentration 4 μg/ml; Southern Biotech) on ice for 30 min. Cells were then washed twice with cold PBS-GA, re-suspended in PBS-GA with Hoechst 33258 (0.5 μg/ml) and analyzed by flow cytometry (LSRII).

For quantification of cell death, cells were harvested with accutase, washed with HBSS with 5uM EDTA and resuspended in HBSS/EDTA. Propidium iodide was added to the final concentration of 2 μg/ml, and cells were analyzed by flow cytometry (LSRII).

2.7. Statistical analysis

Statistical analysis was carried out using the GraphPad Prism 6 software. Experiments were performed in at least three biological replicates, data are presented as mean values ± SEM, and statistical significance was evaluated using ANOVA with differences at $p \leq 0.05$ considered significant.

3. Results

3.1. DR5 receptor-specific ligands are superior to DR4 ligands in triggering apoptotic signaling in cancer cells

In this communication, we aimed to address and compare in detail apoptotic, necrotic and cell death-independent signaling triggered by DR4- or DR5-specific TRAIL ligands in human cancer cells. To achieve this goal, we prepared a set of modified TRAIL receptor-specific ligands containing the extracellular part (amino acids 95–281) of either wild-type (referred to as TST-TRAIL WT) or DR4- and DR5-selective ligand, T4 phage trimerization/stabilization (TRI) motif and the N-terminal TwinStrep tag (TST) (See Fig. S1A and the Methods part). The purified ligands were found to be highly specific and effective in inducing DR4- or DR5-mediated apoptosis in both the predominantly DR4-signaling Ramos Burkitt lymphoma cells and the DR5-expressing Jurkat T cell leukemia cells (Fig. S1B–D).

Colorectal and pancreatic tumors belong to the most studied cancers in the context of TRAIL ligand-induced cell death. We have thus selected colorectal (HT-29) and pancreatic (PANC-1) cancer cell lines for our experiments. The two cell lines express both TRAIL receptors (Fig. 1F, G), with similar cell surface expression of DR4 and lower DR5 expression by PANC-1 cells. Though HT-29 cells feature low total DR4 expression (Fig. 1G), they show a similar DR4 cell surface expression as found in pancreatic PANC-1 cells (Fig. 1F). Both cell lines are also relatively resistant to apoptosis induced by TRAIL alone (HT-29 cells, Fig. 1D, left part; PANC-1, Fig. S2C). Therefore, we included the enhancers of TRAIL-induced apoptosis homoharringtonine (HHT; [24]) and the BH3 analogue ABT-737 [25] for efficient induction of apoptotic signaling. Apoptotic signaling is dependent on efficient activation of caspases, which in the case of TRAIL receptors starts with activation of the initiator caspase-8 within the ligand-receptor-containing activation complex (recently reviewed in [9]). We therefore first examined the time scale of the activation of apical caspase-8, activation of its downstream effector caspase-3, and cleavage of one of its major targets, poly-ADP ribose polymerase (PARP). For enhancement of TRAIL-induced apoptosis, we pre-incubated HT-29 cells (Fig. 1A, B) or PANC-1 cells (Fig. S2A, B) cells with 100 nM HHT for 1 h and then incubated them with 100 ng/ml TST-TRAIL variants for 30 min to 3 h (HT-29 cells) or 30 min to 5 h (PANC-1). In both cell lines we repeatedly observed not only more efficient, but also faster activation of both caspases downstream of DR5-specific ligands. Interestingly, compared to DR5.1, the ligand DR5.2 ligand was much more efficient in inducing the processing of caspases. When using ligands specific for the DR5 receptor, the cleavage of not only PARP (Fig. 1A, B, Fig. S2A, B) but also of the Bid protein, in particular in HT-29 cells with downregulated expression of Mcl-1 (Fig. S3B), was considerably more efficient when compared to the DR4.2 ligand.

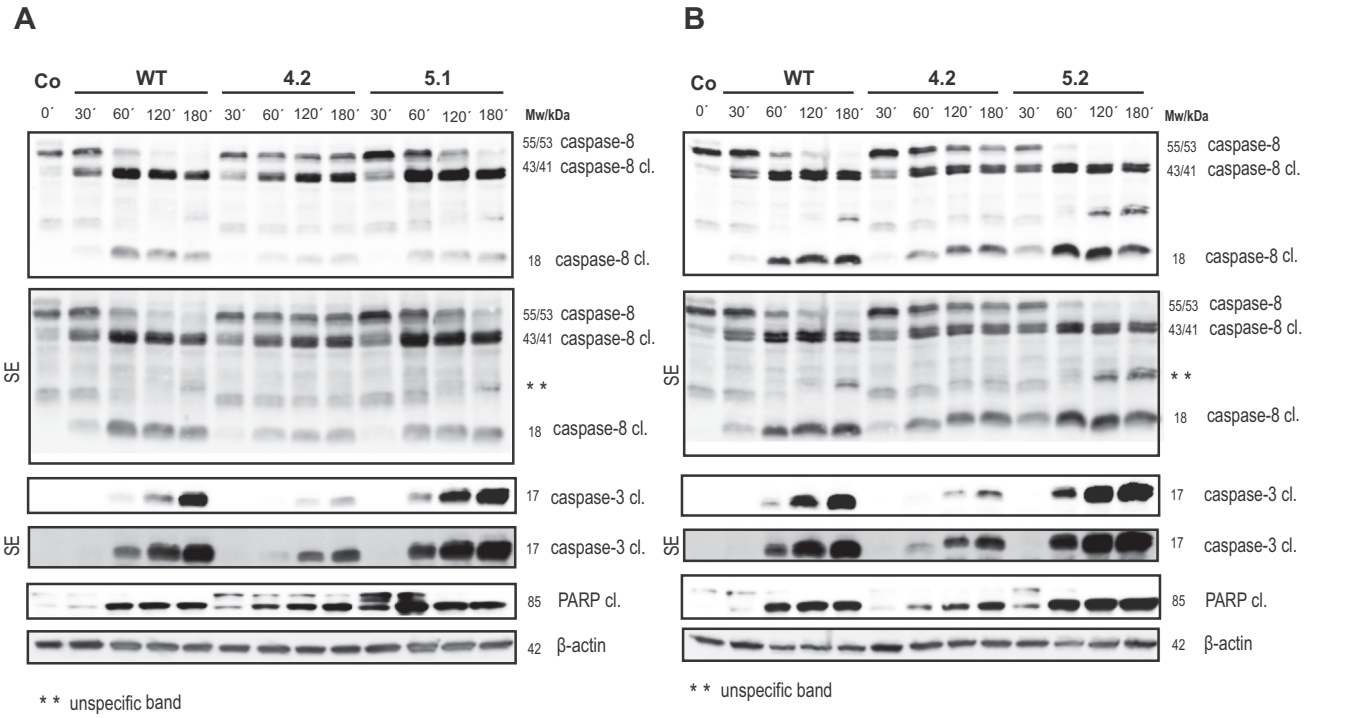
Having documented that pro-apoptotic signaling triggered by DR5-specific ligands was more efficient in activation of caspases than engaging DR4 or in response to wtTRAIL, we next examined whether the enhanced processing of caspases is also reflected in more pronounced induction of apoptosis in target cells. Indeed, we found that the induction of apoptosis in both HT-29 and PANC-1 cells pre-treated with the enhancers HHT or ABT-737 was more robust in HT-29 (Fig. 1C, D) and PANC-1 cells (Fig. 1E) treated with DR5-specific ligands (especially in the case of the DR5.2 ligand). Similarly, using HT-29 cells with downregulated expression of the anti-apoptotic protein Mcl-1 (Fig. S3A), DR5-selective ligands were more efficient in both induction of processing and activation of caspases (Fig. S3B) and in induction of apoptosis (Fig. S3C).

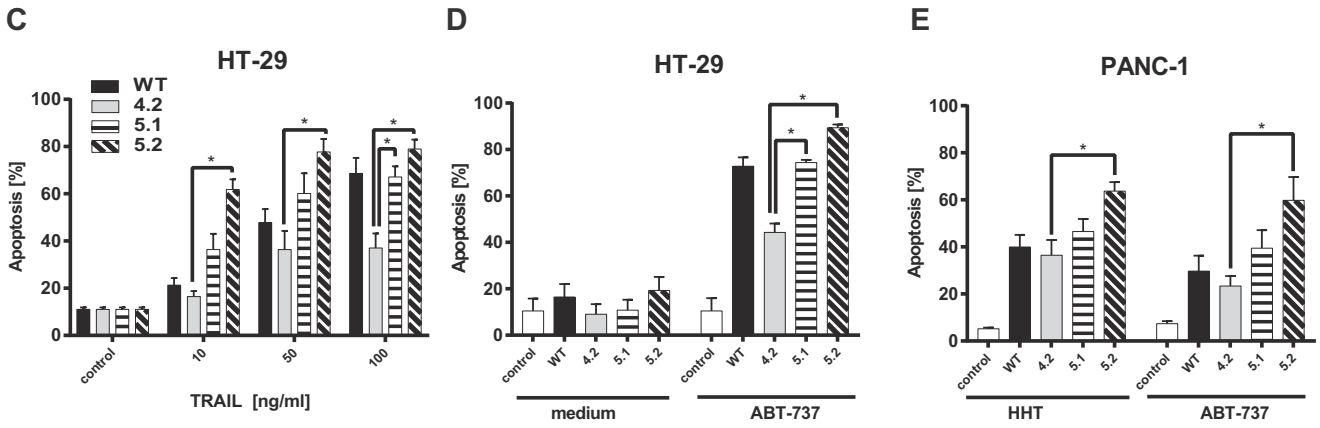
3.2. TRAIL receptor-specific variants do not differ in the formation of death-inducing signaling complex in HT-29 cells

Knowing that DR5-specific ligands are more effective in the induction of caspase processing and induction of apoptosis, and in order to uncover the underlying reasons, we next attempted to dissect the first and essential step in TRAIL receptor signaling, formation and composition of the death-inducing signaling complex (DISC). HT-29 (Fig. 2A) and PANC-1 cells (Fig. 2B) were treated with 1 μg/ml of wt, 4.2, 5.1 and 5.2 TST-TRAIL variants. Affinity purification of DISC components via Strep-tagged TRAIL ligands in HT-29 cells did not show any major differences in either DISC composition or in the kinetics of the formation of the DISC complex (Fig. 2A). However, we noticed that wtTRAIL as well as the DR4-specific ligand attracted FADD in contrast to DR5-selective ligands even during the pre-incubation on ice in both HT-29 and PANC-1 cells (Fig. 2A,B). In contrast to HT-29 cells, we noticed more effective processing of caspase-8 in DISC complexes of PANC-1 cells triggered by DR5-selective ligands (notably DR5.2) (Fig. 2B), which was also reflected in stronger appearance of the processed p43/41 caspase-8 bands in the DR5.2 cell lysates. Interestingly, though DR5-selective ligands triggered comparable or for DR5.1 even less effective DISC formation in HT-29 cells, caspase-8 processing in the cell lysates appears to be more effective.

3.3. DR5 but not DR4 downregulation results in lower susceptibility of colorectal cancer cells to TRAIL

In order to further decipher and confirm the major role of the DR5 receptor in TRAIL-triggered apoptotic signaling, we applied DR4- or DR5-specific lentiviral shRNAs constructs to downregulate their





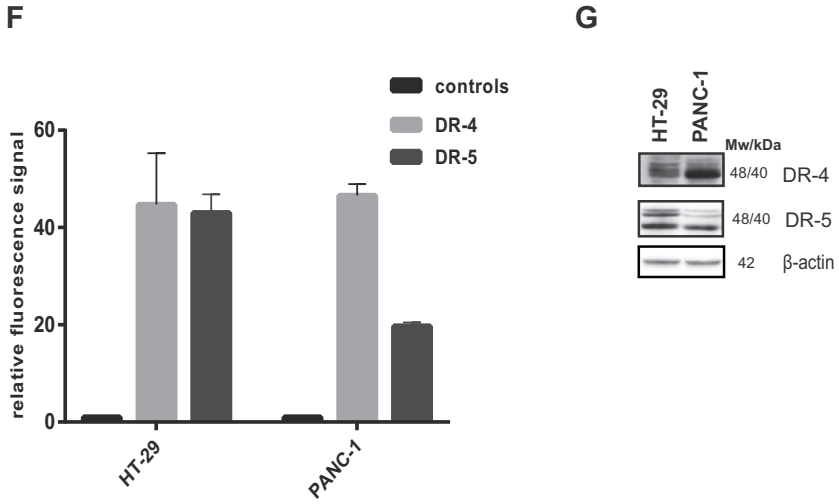


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Fig. 1. DR5 receptor-specific TRAIL ligands effectively induce apoptosis in colorectal and pancreatic cancer cells.
Colorectal HT-29 (A-D) and pancreatic PANC-1 cells (E) were treated with TST-TRAIL variants. Cells were pre-treated with 100 nM HHT for 1 h and then treated with 100 ng/ml of TST-TRAIL-wt, or 4.2, 5.1 and 5.2 ligands for indicated time periods. The samples were then analyzed by western blotting (A, B). SE in Fig. 1 A,B stands for the stronger exposition. For the quantification of apoptosis, HT-29 cells were pre-treated with 100 nM HHT for 1 h (C) or with 20 μM ABT-737 for 4 h (D), and then treated with increasing concentration (C) or with 100 ng/ml (D) of TST-TRAIL-wt, 4.2, 5.1 and 5.2 for 5 h. PANC-1 cells (E) were pre-treated with 100 nM HHT for 1 h or with 20 μM ABT-737 for 4 h and then treated with TRAIL variants at a concentration of 100 ng/ml for 5 h. Cells were evaluated for apoptosis using annexin V-FITC and analyzed by flow cytometry (C-E). Cell surface expression of DR4 and DR5 receptor in HT-29 and PANC-1 cell lines was evaluated by flow cytometry (F). The relative signal is calculated with respect to the control cells stained only with the secondary antibody. The total protein expression of the DR4 and DR5 receptor in the HT-29 and PANC-1 cells was detected by western blotting (G). Data in Fig. 1 C–E were analyzed by ANOVA using GraphPad Prism 6 software and are presented as mean values ± SEM with differences at $p \leq 0.05$ considered as significant. The experiments are representative of at least three biological replicates.

expression in HT-29 cells. Using two different DR4- and two different DR5-directed lentiviral shRNAs, plus the non-targeting shRNA (NT) and empty pLKO1 (EV) controls, we have documented that downregulation of either receptor expression (Fig. 3A, B) resulted in the suppression of DR4- or DR5-selective apoptosis (Fig. 3C). While apoptosis triggered by the wtTRAIL was almost unaffected in HT-29 cells with suppressed expression of DR4, downregulation of DR5 attenuated wtTRAIL-induced apoptosis in these cells, thus supporting the major role of the DR5 receptor in apoptotic signaling in HT-29 cells.

3.4. Both DR4 and DR5 receptor-specific ligands efficiently induce necroptosis in HT-29 cells

Besides inducing apoptosis, activated death receptors can, under specific conditions of suppressed apoptosis, trigger the process of necroptosis. We examined the efficacy of TRAIL receptor-specific ligands in the induction of necroptotic signaling. In these experiments we used HT-29 cells, and induced pro-necroptotic conditions by their pre-treatment with the pan-caspase inhibitor z-VAD and the IAP inhibitor/SMAC mimetic birinapant, which increases the cytosolic pools of RIP1, the essential kinase for the induction of necroptosis from activated death receptors. Necroptosis inhibitors necrostatin-1 (blocks RIP1 kinase activity) and necrosulfonamide (inhibits assembly of MLKL) were also used to document necroptotic cell death. HT-29 cells pre-treated with the combination of birinapant and z-VAD were subsequently treated with increasing concentrations of TST-TRAIL-wt, and dead cells were quantified using propidium iodide staining and flow cytometry. We show that TST-TRAIL-wt can efficiently induce necroptosis of HT-29 cells even at the low concentration of 10 ng/ml, and that this type of cell death can be suppressed by necrostatin (Fig. 4A) as well as the MLKL inhibitor necrosulfonamide (Fig. 4B). Necroptosis was also confirmed by RIP3 kinase-mediated phosphorylation of the MLKL protein and the absence of caspase-3-processed PARP (Fig. 4D). Notwithstanding this, we observed higher efficacy of DR5-specific ligands (notably, again, DR5.2) in the case of low concentration of the TST-TRAIL ligand at 10 ng/ml (Fig. 4C). We did not notice any significant differences between DR4- and DR5-specific ligands in inducing necroptotic cell death at higher, saturating concentrations of the ligands (100 ng/ml) (Fig. 4B).

3.5. DR5 receptor-specific TRAIL ligands efficiently induce NF-κB, p38 and JNK signaling under both apoptotic and necroptotic conditions

In addition to cell death signaling, ligands from the TNF family activate various types of non-apoptotic signaling from their activated receptors, including activation of the NF-κB pathway, MAP and stress kinases and PI3 kinases; further, and under blockage of cells death, even promotion of proliferation, migration and cells survival may occur [26]. The majority of these TRAIL-triggered signaling pathways are activated through secondary cytoplasmic complexes. Under the pro-apoptotic conditions in both HT-29 and PANC-1 or pro-necroptotic conditions in HT-29 cells, we examined the effect of receptor-specific TRAIL variants on the activation of NF-κB, MAP and stress kinases. In general, under pro-apoptotic conditions, the DR4-specific TRAIL variant was less effective in triggering these signaling pathways (Fig. 5A and S4). Notably, activation/phosphorylation of JNK kinases and IκB phosphorylation was very inefficient in TST-DR4.2-treated HT-29 cells and significantly less efficient also in PANC-1 cells. Under necroptotic conditions, these differences among receptor-specific TRAIL variants were blunted in TRAIL-treated HT-29 cells (Fig. 5B), possibly reflecting similar efficacy of wild-type and receptor-specific variants in triggering necroptosis of HT-29 cells.

4. Discussion

The initial discovery and selective cancer cell killing properties of TRAIL have invoked great expectations for this ligand as a new biological anti-cancer agent [3,27]. However, clinical trials in the past two decades, using either recombinant TRAIL or the receptor-specific agonistic antibodies were largely disappointing, with no or minimal effect of these agents on suppression or elimination of various tumors. Though unsuccessful, the outcome from these trials led to the formulation of better and possibly patient-tailored therapeutic protocols, which include the use of novel sensitizers and the discovery and preparation of modified TRAIL-based agents, thus opening a new window of opportunity for its use in cancer therapy [9,28]. Various TRAIL receptor-specific mutants of this ligand belong among these novel formulations of TRAIL. A selective and computer design-based point mutations of TRAIL led to the preparation of highly active and DR4- or DR5-specific variants of the ligand. Based on this knowledge [19,21,23], we prepared modified and highly effective and selective DR4- and DR5-specific ligand variants containing, in addition to the mutated extracellular part of TRAIL, a stabilizing trimerization motif and TwinStrep tag at their N termini for efficient purification and DISC precipitation.

The modified DR4- and DR5-targeted TRAIL variants were, as expected from already published data, highly selective, inducing apoptosis more efficiently than TST-TRAILwt via DR5-signaling in Jurkat T cells, or DR4-signaling in Ramos B cells. As model cell lines for detailed analysis of DR4- vs. DR5-specific signaling, the colorectal HT-29 and pancreatic PANC-1 cells were selected. These two cell lines are resistant to wtTRAIL-induced apoptosis under normal conditions, but can be sensitized to undergo apoptosis by a number of enhancing agents such as the inhibitor of translation homoharringtonine or the Bcl-2/Bcl-X_L inhibitor ABT-737. The initial comparative analysis of TRAIL receptor-specific ligands in colorectal HT-29 cells proved that DR5-specific ligands are more effective than DR4-targeted ligands and even wtTRAIL in the activation of caspases and the ensuing induction of apoptosis of HT-29 cells. Our data thus support other published reports pointing to the preferential use of DR5-mediated signaling in colorectal cancer cells [29,30], but appear to contradict the recently published data from colorectal cells with gene-edited DR4 and DR5 receptors [31].

Cells with inactivated expression of DR4 become resistant to TRAIL or the DR5 peptidomimetic, pointing to DR4 as a main pro-apoptotic receptor. It might be difficult to sort out this apparent controversy, also in light of another current report that, to the contrary, documents that the DR5 receptor is the one that responds to stress triggered by unfolded protein response [32]. However, using our highly specific and effective ligands, we have confirmed their selectivity; we are therefore not convinced that DR4 is the main (or only) pro-apoptotic receptor, at least in the studied cell lines. It is possible that DR4 is in some cells required to support DR5-mediated signaling and thus its deletion could severely compromise DR5-triggered apoptosis. Our data pointing to

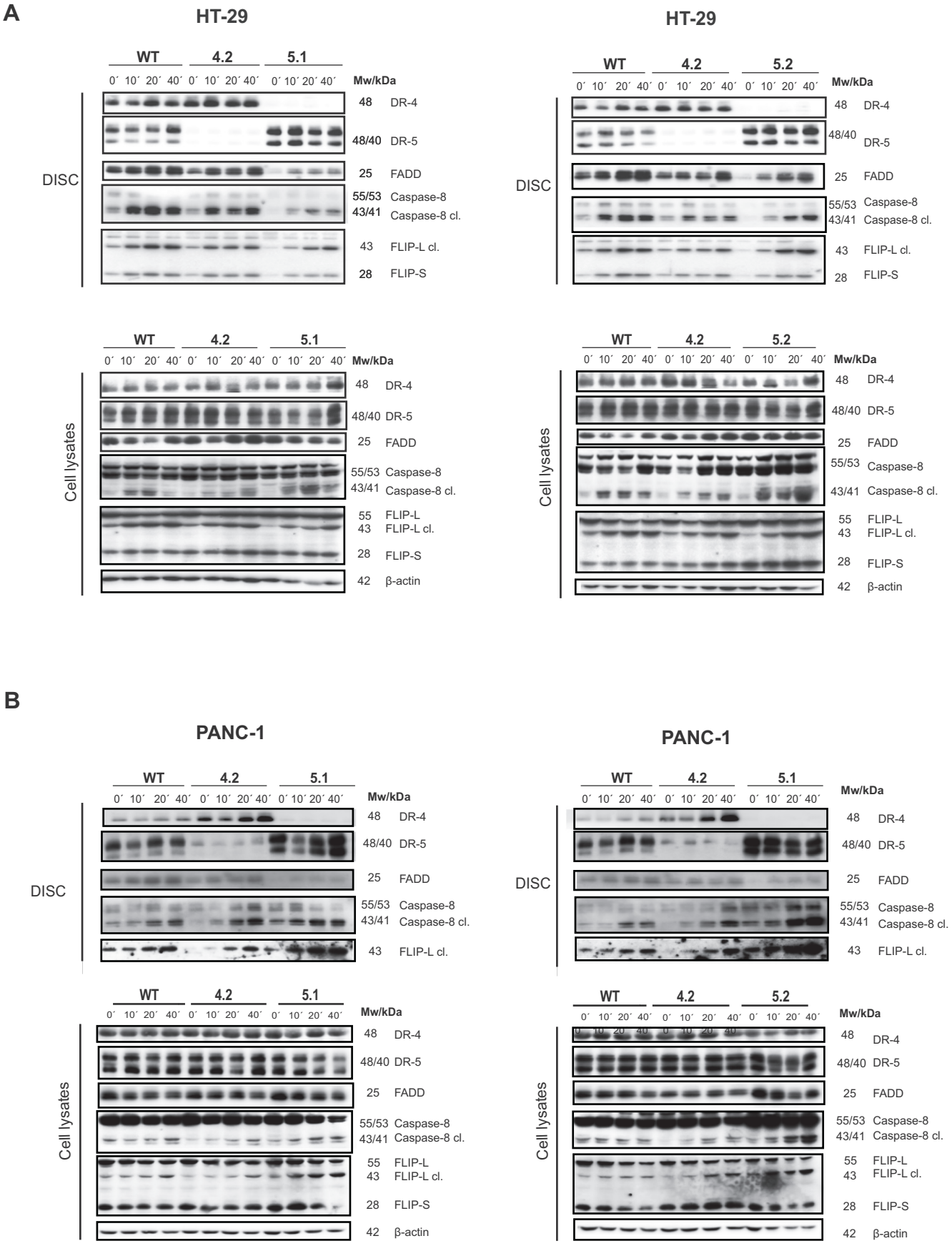

Fig. 2. TRAIL receptor-specific variants induce efficient yet comparable DISC formation in HT-29 and PANC-1 cells.
HT-29 (A) or PANC-1 (B) cells were pre-incubated with 1 μg/ml of TST-TRAILs: wt, 4.2, 5.1 and 5.2 for 15 min on ice. The cells were then either harvested (time point 0 min) or transferred to 37 °C, harvested at specified time periods (10, 20 and 40 min) and lysed. The DISC complexes from the cleared lysates were isolated on Streptactine agarose and analyzed by western blotting. Experiments are representative of two biological replicates.

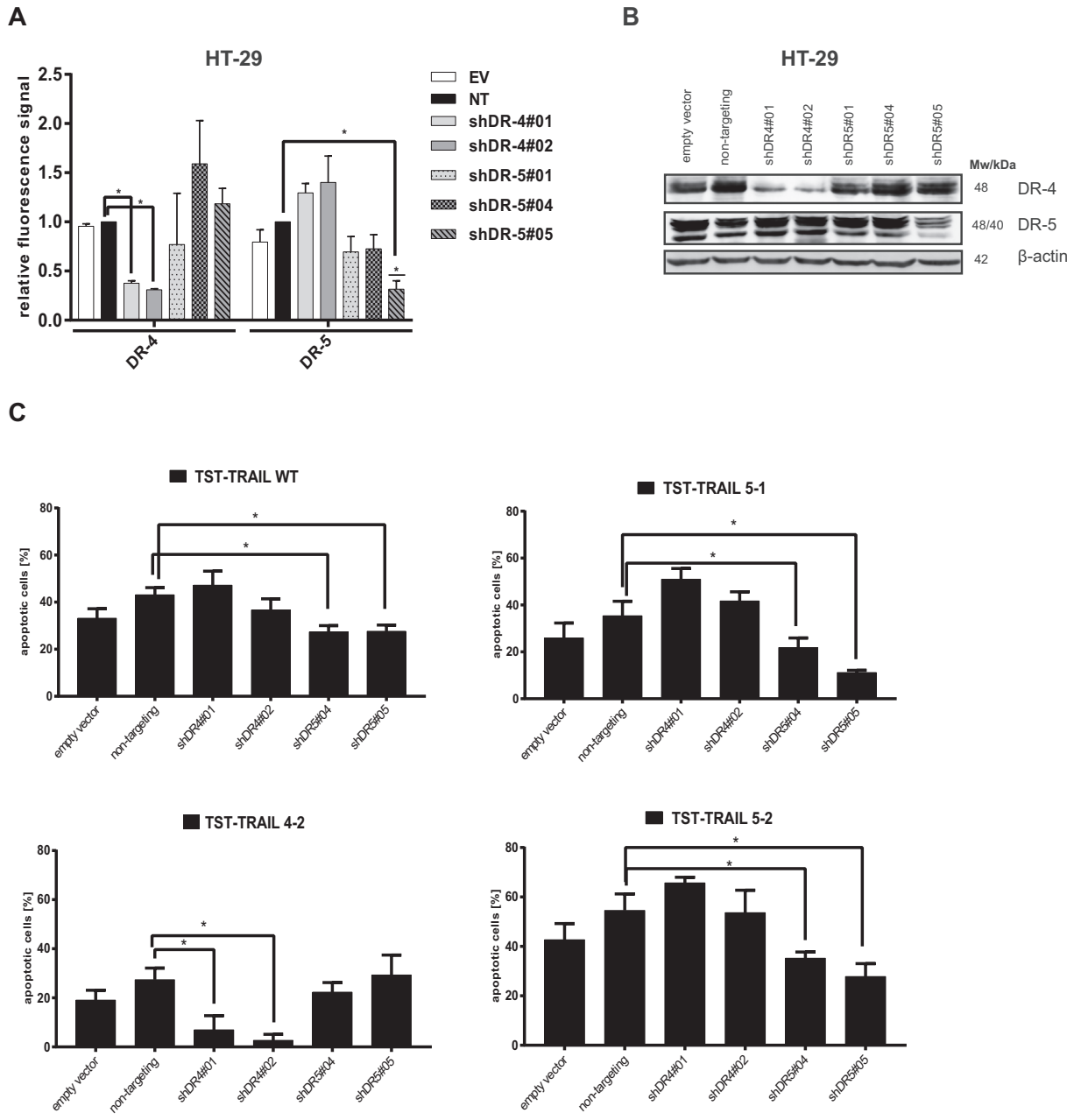

Fig. 3. DR5 but not DR4 downregulation attenuates TRAIL-wt induced apoptosis of HT-29 cells.
shRNA-mediated suppression of the cell surface expression of DR4 and DR5 receptors in HT-29 cells was analyzed by flow cytometry (A). Relative fluorescence signal was normalized to the level of the signal after staining with the secondary antibody only and then against the receptor expression in cells expressing non-targeting (NT) control shRNA. Total intracellular expression of DR4 and DR5 receptor was evaluated by western blotting (B). For the quantification of apoptosis (C), HT-29 were pre-treated with 100 nM HHT for 1 h and then treated with 100 ng/ml TST-TRAIL variants for 3 h. Cells were assessed for apoptosis using annexin V-FITC and the flow cytometry. Data in Fig. 3A,C were analyzed by ANOVA using GraphPad Prism 6 software and are presented as mean values ± SEM with differences at $p \leq 0.05$ considered as significant. The experiments are representative of at least three biological replicates.

significantly more effective DR5-triggered signaling in HT-29 cells, are supported by very efficient vs. almost no activation of JNK signaling by DR5-specific ligands (TST-DR5 vs. TST DR4 ligands), and in the case of the more active DR5.2 ligands strong activation of long pro-apoptotic isoforms of JNK1 [33]. Our data are also supported by the shRNA-mediated knockdown of either DR4 or DR5 expression in HT-29 cells, which in contrast to gene editing-mediated inactivation of these receptors [31], do not entirely eliminate the expression of either of these receptors, and thus allow for their possible cross-talk. In addition, we found that engagement of the DR5 receptor led to an increase in the transcription of the DR5 gene (data not shown), which could lead to increased level of the DR5 receptor and thus to more efficient DR-triggered apoptosis. Moreover, several reports document that ubiquitin-mediated degradation preferentially targets the DR4 receptor, which also likely contributes to the enhanced DR5-mediated apoptotic signaling [34–36].

We also used in our study the PANC-1 pancreatic cancer cells that were reported to employ mainly DR4 in apoptosis induction [16,18,20,21]. Similarly, acute myeloid leukemia cells apparently prefer DR4 receptors for triggering TRAIL-induced apoptosis [17,37]. However, our results from the analysis of TRAIL receptor-specific apoptotic signaling in PANC-1 cells differ from majority of the above-mentioned published data. Both DR5-specific ligands and, notably, more efficient DR5.2 ligand were, despite lower cell surface expression of DR5, significantly more efficient in triggering apoptosis in PANC-1 cells by means of not only TST-TRAIL-wt but also DR4-specific ligand

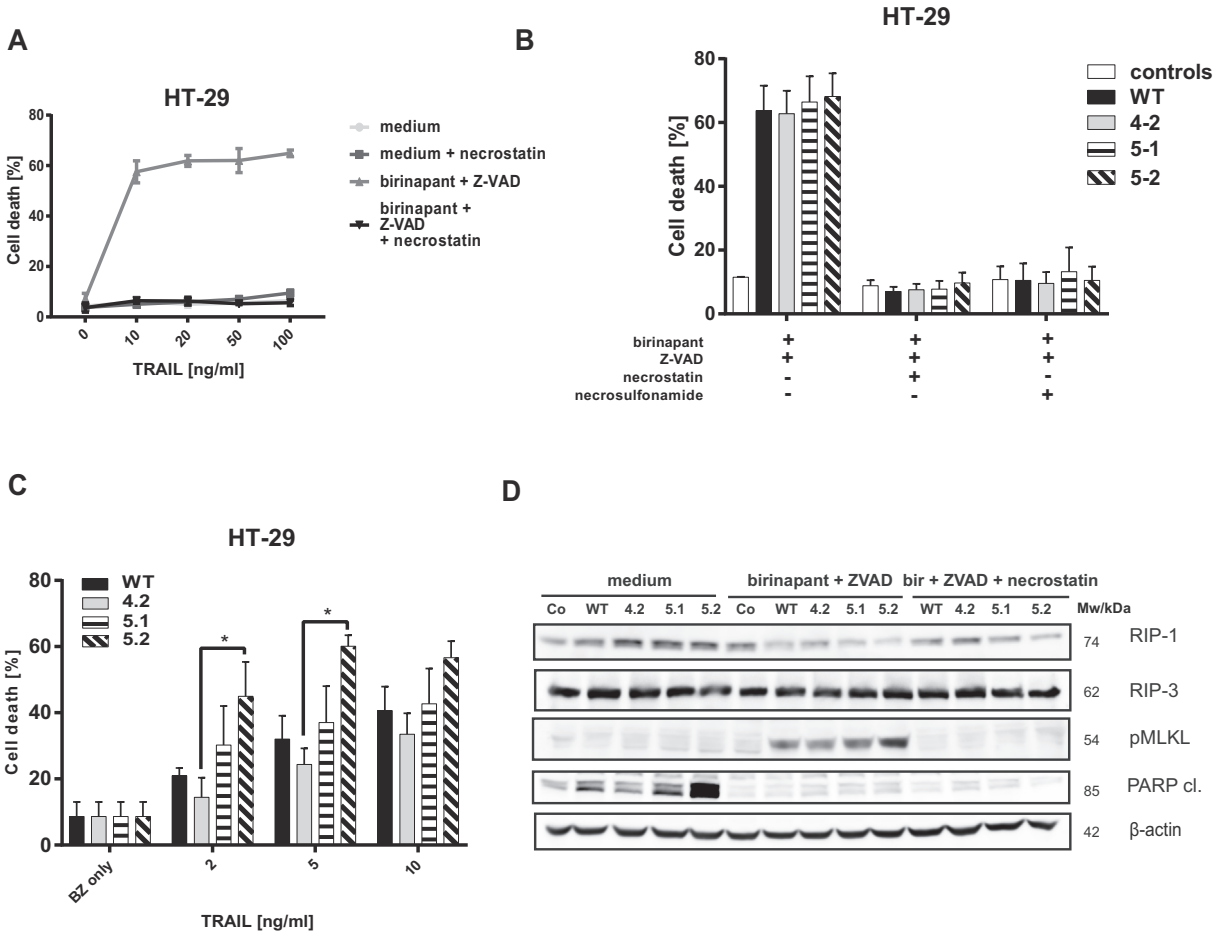

Fig. 4. Both DR4 and DR5 receptor-specific TRAIL ligands efficiently induce necroptosis of HT-29 cells.
HT-29 cells were pre-treated with combinations of 10 nM birinapant plus 50 μM z-VAD or in addition with 50 μM necrostatin for 1 h and then treated with an increasing concentration of TST-TRAIL-wt for 8 h (A). Necroptotic death of HT-29 cells was verified using RIP1 or MLKL inhibitors necrostatine-1 and necrosulfonamide (B). HT-29 were pre-treated with the combinations of 10 nM birinapant, 50 μM z-VAD, 50 μM necrostatin and 20 μM necrosulfonamide as shown for 1 h, and then treated with 100 ng/ml of TST-TRAIL-wt, 4.2, 5.1 and 5.2 for 8 h (B). HT-29 cells were pre-incubated with 10 nM birinapant and 50 μM z-VAD for 1 h and then treated with increasing concentrations of TST-TRAIL ligands for 6 h (C). Cell death was in all cases assessed by propidium iodide staining and flow cytometry. HT-29 cells were pre-treated with the combination of 10 nM birinapant, 50 μM z-VAD and 50 μM necrostatin for 1 h, and then TST-TRAIL ligand variants were added at the concentration of 100 ng/ml for 3 h (D). Samples were analyzed by western blotting. Data in Fig. 4C were analyzed by ANOVA using GraphPad Prism 6 software and are presented as mean values ± SEM with differences at $p \leq 0.05$ considered as significant. The experiments are representative of at least three biological replicates.

DR4.2. The DR5.2 ligand triggered considerable apoptosis in PANC-1 cells even in the absence of a sensitizer/enhancer. Although the initial steps in TRAIL-induced apoptosis involving assembly of DISC and DISC-mediated activation of caspase-8 proceed similarly for the TST-TRAIL-wt and all TRAIL receptors-specific variants in HT-29 cells, DR5-specific ligands are then superior in the amplification of apoptotic signaling. There may be several reasons for these apparent controversies: a) the modified DR5-specific ligands stabilized by the TRI motif promote more effective interactions with DR5 receptors allowing them to transmit efficient apoptotic signaling; b) our ligands do not contain only the generally used 114–281 amino acids region from the extracellular part of TRAIL, but also 20 more membrane-proximal amino acids (i.e. 95–281), which could have an effect on their relative efficacy in inducing apoptotic signaling; and c) the efficacy of DR4- or DR5-triggered apoptotic signaling is apparently positively affected by O-glycosylation of DR5 [38] or N-glycosylation of DR4 [39], and thus our ligands might interact differently than other similar recombinant TRAIL receptor-specific ligands with differently glycosylated cognate receptors. Additional, possibly attractive hypothesis of the reasons for more effective DR5-triggered apoptotic signaling might encompass more efficient chain formation of caspase-8 in DR5-selective DISC complexes with rapid kinetics of caspase-8 processing [40,41]. Our data are also in agreement with recent screenings of pancreatic and colorectal cancer cells for their response of TRAIL receptor-specific variants, pointing to the DR5 preference for PANC-1 cells, although various preferences for DR5- vs. DR4-mediated signaling were reported for different pancreatic cancer cells [30]. Indeed, we found almost equal preference for DR4- and DR5-selective apoptosis with a slight incline into DR5-triggered apoptotic signaling in other pancreatic cancer cell line PaTu (data not shown).

TRAIL death receptors can similarly as other receptors from the TNFR family trigger RIP1/RIP3-dependent necroptosis [42,43]. Under the conditions favoring necroptosis (i.e. inhibiting caspases and the IAP proteins), wtTRAIL, as previously shown [43], induced dose-dependent, necrostatin-1 suppressible necroptosis of HT-29 cells, consistent with previous findings [43]. However, in contrast to apoptosis, all DR4- and DR5-specific ligands were, at saturating concentrations, equally effective in triggering RIP1- and MLKL-dependent necroptosis in HT-29 cells. TRAIL receptor-specific ligands similarly enhanced activation of non-apoptotic signaling, such as triggering phosphorylation of p38 and JNK kinase. Notably, in spite of equal activation of necroptosis at saturating concentration of TST-TRAIL ligands, we observed higher efficacy of the DR5.2 ligand at its lower concentration, likely reflecting its higher binding affinity for the DR5 receptor [23]. One of the reasons could be

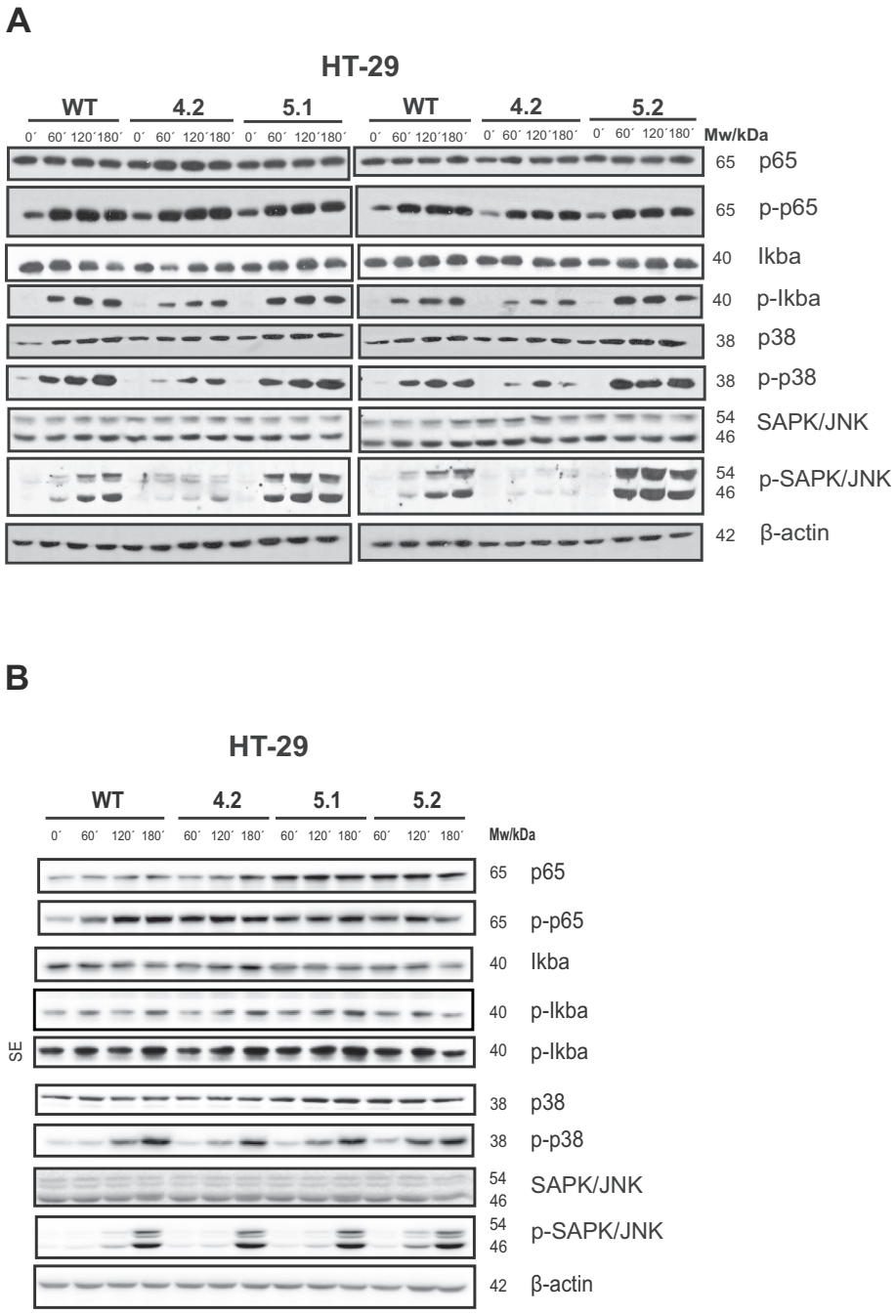

Fig. 5. DR5 receptor-specific TRAIL ligands effectively induce NFκB, p38 and JNK signaling under both apoptotic and necroptotic conditions.
HT-29 cells were treated with 100 ng/ml of TST-TRAIL-wt, 4.2, 5.1 and 5.2 for 60–180 min (A) or were pre-treated with both 10 nM birinapant and 50 μM z-VAD for 1 h and then treated with 100 ng/ml of TST-TRAIL ligand variants for 60–180 min (B). Cell lysates were analyzed by western blotting. SE in Fig. 5B stands for the stronger exposition. Experiments are representative of at least three biological replicates for Fig. 5A and two for Fig. 5B.

related to the endocytosis and endosomal acidification [44] in a process of pro-death signaling from TRAIL receptors. In apoptotic signaling, dynamin 1-mediated endocytosis likely attenuates TRAIL-mediated apoptosis [45], but it apparently does not affect TRAIL-triggered necroptosis [46].

In conclusion, using a number of approaches, we have shown that TRAIL receptor-specific pro-apoptotic signaling in colorectal and in pancreatic cancer cells could, in contrast to the recent notion, largely rely on the DR5 death receptor, and that this dependence is blunted in TRAIL-triggered necroptosis.

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Human Embryonic and Induced Pluripotent Stem Cells Express TRAIL Receptors and Can Be Sensitized to TRAIL-Induced Apoptosis

Vladimir Vinarsky,[1] Jan Krivanek,[1] Liina Rankel,[2] Zuzana Nahacka,[2] Tomas Barta,[1] Josef Jaros,[1] Ladislav Andera,[2] and Ales Hampl[1,3]

Death ligands and their tumor necrosis factor receptor (TNFR) family receptors are the best-characterized and most efficient inducers of apoptotic signaling in somatic cells. In this study, we analyzed whether these prototypic activators of apoptosis are also expressed and able to be activated in human pluripotent stem cells. We examined human embryonic stem cells (hESC) and human-induced pluripotent stem cells (hiPSC) and found that both cell types express primarily TNF-related apoptosis-inducing ligand (TRAIL) receptors and TNFR1, but very low levels of Fas/CD95. We also found that although hESC and hiPSC contain all the proteins required for efficient induction and progression of extrinsic apoptotic signaling, they are resistant to TRAIL-induced apoptosis. However, both hESC and hiPSC can be sensitized to TRAIL-induced apoptosis by co-treatment with protein synthesis inhibitors such as the anti-leukemia drug homoharringtonine (HHT). HHT treatment led to suppression of cellular FLICE inhibitory protein (cFLIP) and Mcl-1 expression and, in combination with TRAIL, enhanced processing of caspase-8 and full activation of caspase-3. cFLIP likely represents an important regulatory node, as its shRNA-mediated down-regulation significantly sensitized hESC to TRAIL-induced apoptosis. Thus, we provide the first evidence that, irrespective of their origin, human pluripotent stem cells express canonical components of the extrinsic apoptotic system and on stress can activate death receptor-mediated apoptosis.

Introduction

Human embryonic stem cells (hESC) originating from the inner cell mass of human blastocysts and human-induced pluripotent stem cells (hiPSC) produced by forced reprogramming of somatic cells by gene expression represent two types of human pluripotent stem cells with tremendous potential in various biomedical applications, including cell therapy, disease modeling, and drug development [1–4]. Although these types of human pluripotent stem cells can indefinitely proliferate in culture, unlike transformed cancer cells, they are prone to demise by apoptosis [5–7]. Both hESC and hiPSC express, and if necessary also employ, key canonical components and regulators of apoptotic signaling [8,9]. DNA damage, ectopic expression of oncogenes such as c-Myc, heat shock, viral infection, or even cell dissociation can trigger intrinsic apoptotic signaling that is largely dependent on pro-apoptotic proteins from the Bcl-2 family [5–7,10–12]. However, hESC and hiPSC can be at least partially protected against stress-induced apoptosis by a number of treatment modalities, such as addition of growth factors and/or inhibitors of ROCK kinase to culture media or by ectopic expression of anti-apoptotic Bcl-2 proteins [13–18]. Another level of anti-apoptotic protection in hESC involves increased expression of survivin, an anti-apoptotic member of the inhibitor of apoptosis (IAP) family that also contributes to teratoma formation [19,20]. In summary, elements of the intrinsic apoptotic pathway are clearly active in both hESC and hiPSC and are employed to regulate their homeostasis.

In addition, in virtually all somatic cells, apoptosis can also be mediated by the extrinsic pathway that is triggered by so-called death ligands from the tumor necrosis factor (TNF) family [TNFα, FasL, and TNF-related apoptosis-inducing ligand (TRAIL)] and their corresponding death receptors present on the cell surface [21,22]. Apoptotic signaling from death receptors relies on ligand-triggered clustering of receptors via their intracellular protein–protein interaction region called the death domain, followed by

[1]Department of Histology and Embryology, Faculty of Medicine, Masaryk University, Brno, Czech Republic.
[2]Laboratory of Cell Signaling and Apoptosis, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic.
[3]International Clinical Research Center, St. Anne's University Hospital, Brno, Czech Republic.

formation of the Death-Inducing Signaling Complex (DISC), a multiprotein platform that is critical for the proximity-based auto-processing and activation of the main initiator caspase-8 (recently reviewed in [23,24]). Activated caspase-8, and in some cases also caspase-10, then cleaves its cellular targets, most notably the effector caspase-3, the mitochondrial apoptotic signaling activator Bid (into truncated Bid or tBid), and the caspase-8 antagonist cellular FLICE inhibitory protein (cFLIP), resulting in cleavage of poly (ADP-ribose) polymerase (PARP), a well-established marker of ongoing apoptosis [25,26]. In addition to caspase-dependent apoptosis, under certain circumstances, death receptors can trigger a specific receptor-interacting protein (RIP)1/RIP3-dependent form of programmed necrosis called necroptosis [27,28]. Importantly, normal mesenchymal stem cells, progenitor cells, and terminally differentiated cells are resistant to death receptor-induced pro-death signaling [29–31]. In these cells, ligand-activated receptors may induce a number of other signaling events, for example, activation of the canonical NFκB pathway, mitogen-activated protein (MAP) and stress kinases, and the P3K/Akt axis, and can even enhance macroautophagy [32–34]. Considering the ultimate outcome of death receptor-induced pro-apoptotic signaling, both its initial and follow-up steps should be delicately regulated. At the proximal DISC node, expression levels of the caspase-8 antagonist cFLIP and also the efficacy of caspase-8 clustering and its stability have a pronounced impact on the robustness of pro-apoptotic signaling from the activated Fas/CD95 or TRAIL receptors [35–39]. More distally, efficient activation of effector caspases can be blunted at the mitochondria by blocking tBid-mediated amplification of apoptotic signaling or by competitive inhibitors of activated caspases from the IAP family [20,40,41].

As indicated earlier, in general, only damaged, transformed, or unneeded cells are induced to undergo apoptosis by death ligands, and TRAIL was brought to the forefront for its potential use in anti-tumor therapy [42,43]. hESC, and particularly hiPSC, may possess and/or develop characteristics that are typical of damaged or transformed cells. Though the human pluripotent stem cells express all canonical components of the extrinsic apoptotic signaling, they are, as we document, resistant to TRAIL. However, we show that on stress such as proteo-synthesis inhibition, both hESC and hiPSC become sensitized to TRAIL-induced apoptosis and we point to cFLIP as an essential molecule conferring TRAIL resistance in hESC.

Materials and Methods

Cultivation and treatment of hESC and hiPSC

Two hESC lines (CCTL 12 and CCTL 14) [44] between passages 25–80, and one hiPSC cell line (clone 4) [45] between passages 50–80, were used for these experiments. Colonies of hESC and hiPSC were either co-cultivated with mitotically inactivated mouse embryonic fibroblasts (MEFs; mouse strain-CF1; density 24,000 cells/cm^2) as previously described [46] or grown on an MEF-derived extracellular matrix (ECM). Cultivation on ECM required MEF-conditioned hESC medium (CM) and was used during various treatments of the stem cells to avoid the bystander effect of MEFs.

ECM was prepared as follows: MEFs (mouse strain CF1, density 24,000 cells/cm^2) were seeded on gelatin-coated dishes and grown for 5 days. Cells were then lysed on the plates by 0.5% deoxycholate in 10 mM Tris-HCl, pH 8.0, and washed five times with phosphate-buffered saline (PBS) without Mg^{2+} and Ca^{2+} (pH 7.4) at 4°C. Isolated ECM was stored in PBS at 4°C for approximately 1 week. For preparation of MEF-conditioned hESC medium, hESC medium was incubated with mitotically inactivated MEFs for 24 h, collected, supplemented with 10 ng/mL FGF2 (#100-18B; Pepro Tech) and 2 mM L-glutamine (#25030-24; GIBCO), filtered, and stored for approximately 1 week at 4°C.

For treatment with human recombinant TRAIL (P-008; Apronex), homoharringtonine (HHT) (H0635; Sigma-Aldrich), pan caspase inhibitor Z-VAD-FMK (C2105; Sigma-Aldrich), caspase-8 inhibitor Z-IETD-FMK (550380; BD Pharmingen), cycloheximide (CHX) (C1988; Sigma-Aldrich), and necrostatin-1 (N9037; Sigma-Aldrich), hESC or hiPSC were allowed to grow on ECM in CM medium for 3–5 days after passage. The medium was changed 24 h before treatment, and the appropriate reagents were added to the media at the desired concentrations. To harvest the treated cells, colonies were washed with PBS and incubated for 2 min with 0.5 mM EDTA in PBS at 37°C. Cells were detached from the surface by gentle pipetting and collected in cold PBS. For detection of apoptosis, both adherent and floating cells were collected.

Flow cytometric analysis of death receptor expression

Adherent cells were harvested and incubated in PBS containing 20% human AB serum (Faculty Hospital, Brno, Czech Republic) and 0.2% cold water fish gelatin (G7765; Sigma-Aldrich) for 10 min. Cells were then washed in PBS-G buffer (PBS + 0.2% cold fish gelatin and 0.1% NaN_3) and incubated on ice for 30 min with primary antibodies specific for DR4 (DR-4-02; Exbio Praha), DR5 (DR5-01-1; Exbio Praha), DcR1 (HS301; Enzo LS), DcR2 (HS402; Enzo LS), TNFR1 (#16803; RnD systems), and Fas (DX2; ENZO LS), or isotype control (P3×, kindly provided by Prof. Peter W. Andrews [Department of Biomedical Science, University of Sheffield]). Cells were then washed twice with PBS-G and incubated with R-phycoerythrin-conjugated secondary antibody (1070-09; Southern Biotech) for 30 min on ice. Cells were washed twice with PBS-G, and receptor expression was analyzed by flow cytometry (FACS Canto II; BD Biosciences). We analyzed 20,000–30,000 cells for each sample. The level of receptor expression was expressed as the ratio of the median fluorescence index (MFI) of specific antibody to the MFI of isotype-stained control using FlowJo software (www.flowjo.com).

Detection and quantification of apoptosis and cell death by flow cytometry

Floating and adherent cells were harvested, washed with PBS, and permeabilized in 90% methanol for 30 min at 4°C. Cells were washed in incubation buffer (0.5% BSA in PBS) and then incubated with the incubation buffer containing RNAse-A (0.02 mg/mL) (Boehringer) at 37°C for 30 min. After washing with incubation buffer, cells were incubated with a primary antibody that was specific for the cleaved form of PARP (#9541; Cell Signaling Technologies) at room temperature for 1 h. Cells were then washed twice in the

incubation buffer and incubated with secondary Alexa Fluor 488-conjugated antibody (A11008; Invitrogen) and propidium iodide in the dark at room temperature for 30 min to allow simultaneous detection of DNA content and the protein of interest. The fluorescence intensity of the population of mononuclear cells (gated using FSC, SSC) was assessed by flow cytometry (FACS Canto II; BD Biosciences). Cell death was assessed by DNA content analysis, by measuring the population of subG1 DNA content. Apoptosis induction was determined as the percentage of cells positive for the cleaved form of PARP. More than 10,000 cells were analyzed for each sample using FlowJo software. Specificity of the antibody recognizing the cleaved form of PARP was verified using western blotting (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd).

Western blotting

Harvested cells were washed thrice with PBS, lysed in lysis buffer [50 mM Tris-HCl (pH 6.8), 1% sodium dodecyl sulfate (SDS), and 10% glycerol], adjusted to a protein concentration of 1 mg/mL, and stored at −70°C until use. Western blot analysis was performed as previously described [46]. The following primary antibodies were used: caspase-3 (#9662), caspase-8 (#9746), phosphorylated NFκB p65 (Ser536) (#3033), phosphorylated Erk1/2 (MAPK) (Thr202/Tyr204) (#9101), Bid (#2002), Bcl-xL (#2764) (all from Cell Signaling Technologies), Caspase-10 (#M059-3; MBL International Corporation), FADD (#556402; BD Pharmigen), cFLIP (alx-804–428; Alexis), Bax (2281-MC-100; Trevigen), Mcl-1 (M8434; Sigma-Aldrich), and XIAP (48-hILP-XIAP; BD Transduction Laboratories). A protein ladder was used to identify the molecular weights of the analyzed proteins (#26619; Thermo Scientific).

Preparation of hESC with down-regulated expression of cFLIP or Mcl-1

HEK293T cells were transfected with the lentivirus packaging plasmids pMD2G, psPAX (Addgene), and pLKO.1 expressing short hairpin RNA (shRNA) against cFLIP (TRCN0000007229, shFLIP1; TRCN0000007230, shFLIP2), Mcl-1 (TRCN0000005515, shMcl-1-2; TRCN0000005517, shMcl-1-4; TRCN0000005518, shMcl-1-5), or nontargeting shRNA SHC002 (all from Sigma-Aldrich). After 2 days, the lentiviral particles were purified from the supernatant using PEG/it Virus Precipitation Solution (LV810A-1, SBI). The hESC (CCTL14) were transduced at a multiplicity of infection (MOI) of 5, selected in medium containing puromycin (3 μg/mL) for 4 days, and analyzed by western blotting for expression of the target gene protein. Survival, expression of pluripotency markers (SSEA-3, SSEA-4, and TRA 2–54), and expression of TRAIL receptors were monitored for approximately 20 passages and were identical to wild-type cells. The cells in passages 5–15 were used for all analyses.

Statistical analysis of FACS data

Data obtained for MFIs and the frequencies of apoptotic cells containing cleaved PARP are presented as means ± standard error of means (SEM). Each bar represents at least three independent experiments. Statistical significance of the data was assessed by Student's unpaired *t*-test using Graph Pad Prism. Values of $*p < 0.05$ were considered significant, $**p < 0.01$ very significant, and $***p < 0.001$ extremely significant.

Results

Human pluripotent stem cells express TRAIL receptors, but are resistant to TRAIL-induced apoptosis

Death receptors from the TNFR family, namely TNFR1, Fas/CD95, and the death domain-containing TRAIL receptors TRAIL-R1/DR4 and TRAIL-R2/DR5, are major inducers of both extrinsic apoptosis and necroptosis in mammalian cells, and their expression levels and regulation of their pro-apoptotic activation represent a fine balance between cell survival and death. Differentiated and somatic stem cells and their progenitors are known to express various levels of these receptors, but no such information is available for human pluripotent stem cells.

In our initial experiments, we measured cell surface expression of these receptors in two hESC lines and one hiPSC line. Representative histograms for hESC (line CCTL14) and hiPSC (clone 4) are shown in Fig. 1A. Figure 1B shows the average of normalized median fluorescence intensity (MFI) obtained from at least three independent repeats for each cell line. hESC and hiPSC exhibited the same pattern of receptor expression, with relatively strong expression of both pro-apoptotic TRAIL receptors, lower expression of TNFR1, and minimal to no expression of the Fas/CD95 receptor. Among inhibitory TRAIL decoy receptors, only TRAIL-R4/DcR2 was significantly expressed on both hESC and hiPSC (Fig. 1A, B). Expression of mRNAs for the respective receptors as determined by qRT PCR (data not shown) was in full consonance with the flow cytometry findings cited earlier. The expression of death receptors is a key prerequisite for effective apoptotic signaling, but the DISC-forming proteins and other down-stream molecules are also required. Figure 1C shows that both hESC and hiPSC expressed significant quantities of DISC components: the adapter protein FADD, the initiator caspases 8 and 10, and their antagonist cFLIP. Similarly, both cell types expressed the BH3-only protein Bid, which transduces pro-apoptotic death receptor signaling to mitochondria, effector caspase-3, and pro- and anti-apoptotic members of the Bcl-2 family (Fig. 1C). It should be noted that levels of initiator caspase-8 and -10 in hESC and hiPSC were similar to those in cultured adult human dermal fibroblasts (AHDF), whereas levels of anti-apoptotic Bcl-2 family proteins and cFLIP were lower (data not shown).

To evaluate the functionality of the extrinsic apoptotic pathway, we first exposed hESC and hiPSC to a wide range of concentrations (0–1 μg/mL) of human recombinant TRAIL for 24 h. Both cell types seemed to be refractory to even the highest concentrations of TRAIL, as demonstrated by their unchanged morphology (data not shown). For further experiments, we used a single concentration of TRAIL (200 ng/mL), which was able to induce massive apoptosis in TRAIL-sensitive cells such as colorectal cancer cell lines DLD-1 or Colo206F after 6 h of treatment [47]. This 6-h treatment did not evoke apoptosis in either cell type, as determined by the unchanged cell morphology (Fig. 2A) and the absence of cleavage of poly (ADP-ribose) polymerase (PARP) (Fig. 2B).

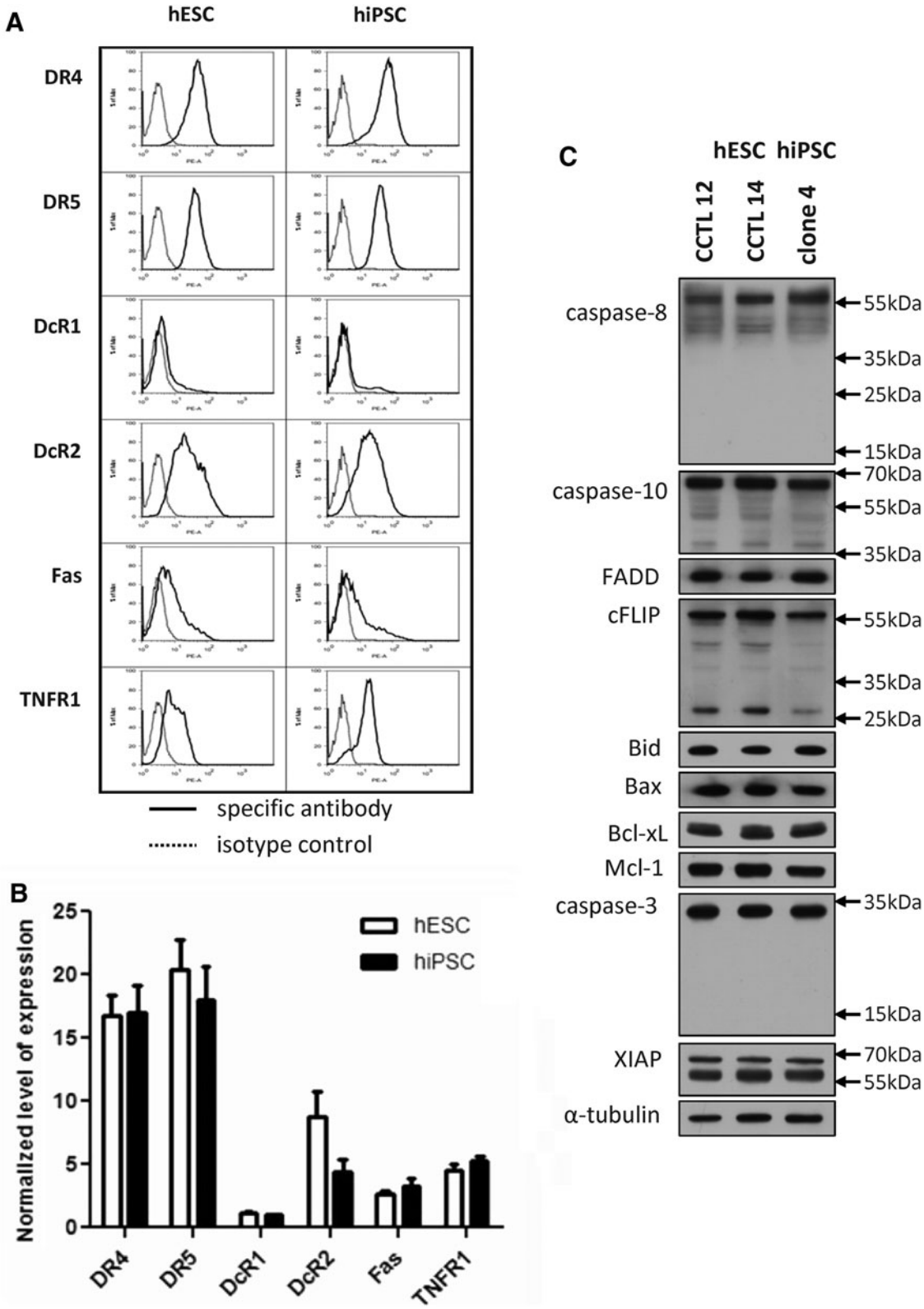

FIG. 1. Expression of extrinsic apoptotic pathway components in human embryonic stem cells (hESC) and human-induced pluripotent stem cells (hiPSC). **(A)** Representative histograms of death receptor expression in hESC (line CCTL14) and hiPSC (clone 4) as determined by flow cytometry. *Solid line*, specific antibody; *dashed line*, isotype control. **(B)** Quantification of levels of expression of death receptors in hESC (CCTL 14) and hiPSC (CCTL12) as determined by flow cytometry. Medians of fluorescent intensity were normalized to the isotype control. Each bar represents the mean of $n > 3$ experiments. Error bars show standard error of mean (SEM). **(C)** Western blot analysis of the expression of intracellular components of the extrinsic apoptotic pathway and selected regulators of apoptosis in two hESC cell lines (CCTL12, CCTL14) and one hiPSC line (clone 4). Alpha tubulin was used as a loading control.

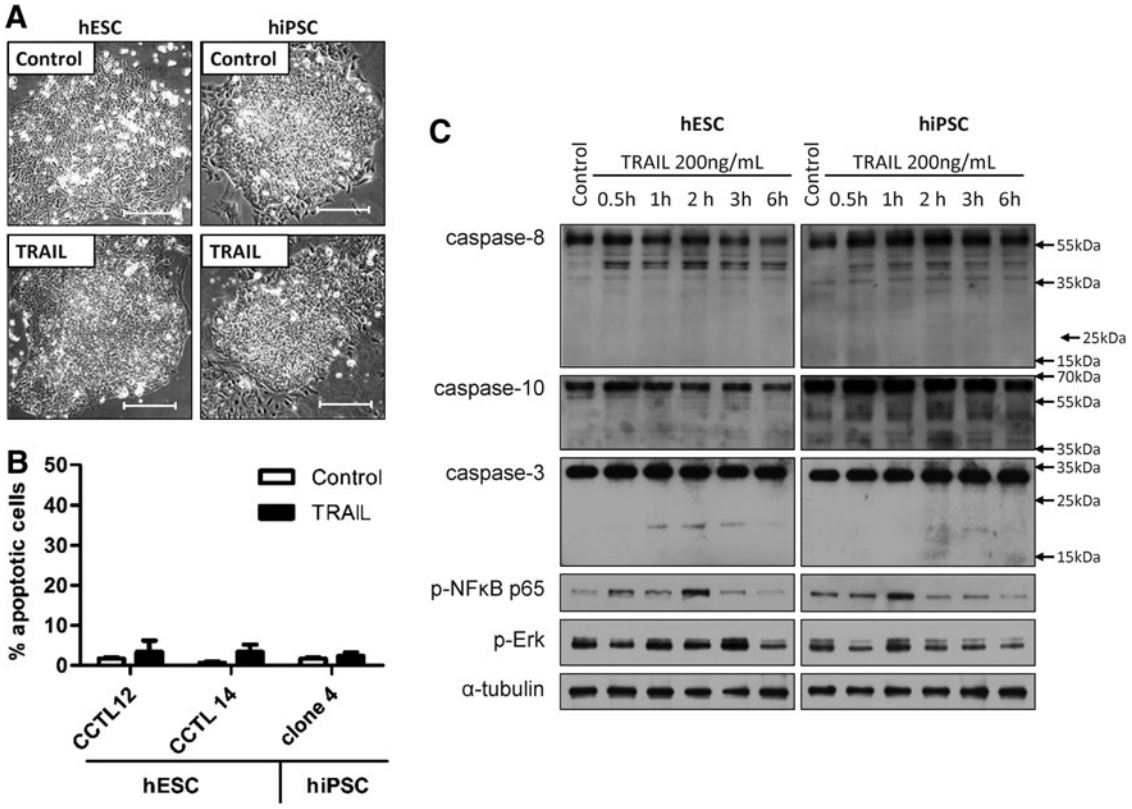

FIG. 2. TRAIL does not induce apoptosis in hESC and hiPSC. The hESC and hiPSC were treated with 200 ng/mL human recombinant TRAIL (TRAIL) or left untreated (control). **(A)** The morphology of colonies and cell detachment were observed by light microscopy after 24 h of treatment. Bar, 200 μm. **(B)** Induction of apoptosis was determined by staining with an antibody that was specific to cleaved poly (ADP-ribose) polymerase (PARP). The percentage of cells that stained positive for cleaved PARP (% apoptotic cells) in control and TRAIL-treated cells after 6 h of treatment was determined by flow cytometry. Each bar represents the mean of $n = 3$ experiments; error bars show ± SEM. **(C)** Western blot analysis of activation of the extrinsic apoptotic pathway, NFκB pathway, MAPK/ERK pathway, and caspase 3 after 30 min and 1, 2, 3, and 6 h of TRAIL treatment. Alpha tubulin was used as a loading control.

This was also true when exposure to TRAIL was prolonged to 24 and 48 h (Supplementary Fig. S2). However, the activation of TRAIL receptors in the first hours of treatment seemed to be partially functional, because in TRAIL-treated cells, the initiator caspase-8 became preactivated and was cleaved to its p43/41 interforms. We also observed caspase-3 cleavage to the p20 preactive fragment, but the p16 and p18 active fragments were not produced (Fig. 2C). In addition to the partial processing of caspases, we also observed time-dependent changes in phosphorylation of the p65 subunit of NFκB and MAP kinases Erk1/2, indicating that nonapoptotic signaling from the activated TRAIL receptors is functional in human pluripotent stem cells (Fig. 2C).

The protein synthesis inhibitor HHT sensitizes human pluripotent stem cells to TRAIL-mediated apoptosis

TRAIL-induced apoptosis can be regulated at several levels: the activated receptors, the mitochondria, and post-mitochondrial signaling. Most nontransformed cells can be sensitized to TRAIL-induced apoptosis by various agents that, in principle, bring down either one or several of the safeguards and/or shift the cell status toward apoptosis. We recently discovered that the natural alkaloid HHT, which inhibits translation, is such a sensitizer [48]. When applied to hESC and hiPSC, HHT alone had almost no effect on cell survival as evidenced by no change in cell morphology (Fig. 3A) and only a minimal increase in the proportion of cells containing cleaved PARP (Fig. 3B), similar to the effects of TRAIL. However, both parameters of ongoing apoptosis, cell detachment, and number of cells with cleaved PARP increased dramatically when these compounds were applied in combination (Fig. 3A, B). In full consonance with these findings, western blot analysis revealed that, although individually, TRAIL and HHT induce detectable processing of the initiator caspase-8 to the p43/41 interforms and the effector caspase-3 to its p20 interform, and only co-treatment unleashes full activation of caspase-8 (monitored by its cleaved p18 form) and caspase-3 (monitored by active p18 and p16 forms) (Fig. 3C). Since one of the previously observed effects of HHT is inhibition of protein synthesis [48], we repeated the same set of analyses in cells, in which

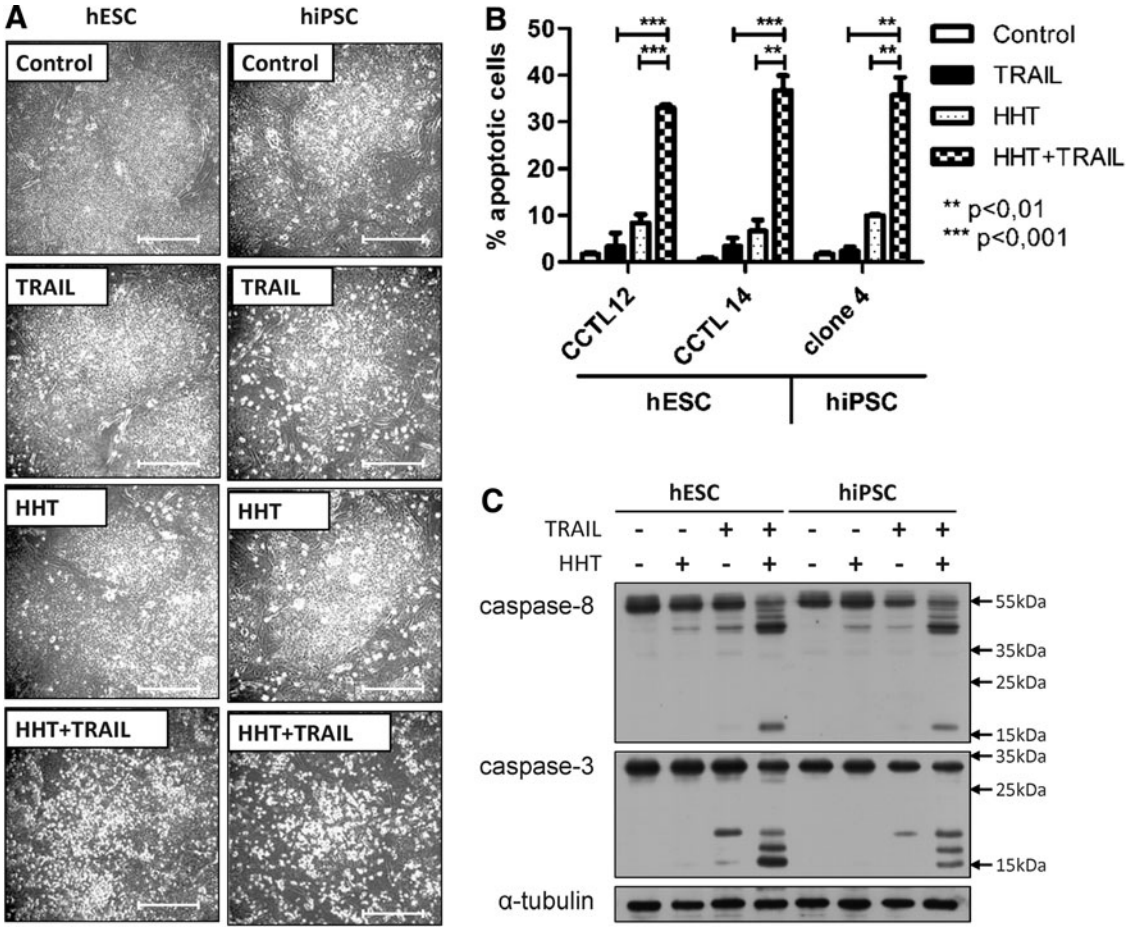

FIG. 3. The protein-synthesis inhibitor homoharringtonine (HHT) sensitizes hESC and hiPSC to TRAIL-induced apoptosis. HESC and hiPSC were left untreated (control), treated with 50 nM HHT or 200 ng/mL human recombinant TRAIL, or pretreated with 50 nM HHT for 1 h followed by 200 ng/mL human recombinant TRAIL treatment (HHT + TRAIL). **(A)** The morphology of colonies and cell detachment of control, HHT-, TRAIL-, or HHT + TRAIL-treated cells was observed by light microscopy after 3 h of treatment. Bar, 200 μm. **(B)** Induction of apoptosis was determined by staining with an antibody that was specific to cleaved PARP. The percentage of cells that stained positive for cleaved PARP (% apoptotic cells) in control, HHT-, TRAIL-, and HHT + TRAIL-treated cells after 6 h of treatment was determined by flow cytometry. Each bar represents the mean of $n = 3$ experiments; error bars show ± SEM. **(C)** Western blot analysis of activation of the extrinsic apoptotic pathway in control, HHT-, TRAIL-, and HHT + TRAIL-treated cells after 3 h of treatment. Alpha tubulin was used as a loading control.

protein synthesis was inhibited by the classic protein inhibitor, CHX. The resulting data that are summarized in Supplementary Fig. S3A–C fully match those obtained with HHT.

It is of note that both the pancaspase inhibitor Z-VAD-FMK and the specific caspase-8 inhibitor Z-IETD-FMK suppressed cell death (with Z-IETD-FMK being less efficient) in HHT and TRAIL co-treated hESC, indicating its dependence on caspase-8 (Supplementary Fig. S4A, B). At the molecular level, treatment with both caspase inhibitors led to reduced cleavage of initiator caspase-8 to its p43/41 interforms and abolished production of its p18 form. Cleavage of caspase-3 to its active p18 and p16 forms was also significantly reduced (Supplementary Fig. S4C), indicating that execution of cell death induced by TRAIL in HHT-sensitized cells is caspase-8 dependent.

Taken together, our findings demonstrate that both types of human pluripotent stem cells can be made sensitive to TRAIL-induced apoptosis by a single compound, a protein synthesis inhibitor. We took advantage of this observation to probe for factors that inhibit the extrinsic pathway under normal conditions. We first analyzed whether HHT treatment affected cell surface expression of the TRAIL receptors. As shown in Fig. 4A, exposure of both hESC and hiPSC to 50 nM HHT for 4 h resulted in subtle changes to TRAIL receptor expression with only weak up-regulation of DR5 being reproducible. Next, we examined whether HHT treatment modified the quantity of major apoptosis-activating or apoptosis-regulating proteins expressed in hESC or hiPSC. Treatment of the cells with 50 nM HHT for approximately 4 h did not change the expression of major initiator caspases-8 and -10, pro- and anti-apoptotic proteins Bax and Bcl-xL, and the caspase-3/-9 competitive inhibitor XIAP (Fig. 4B). On the other hand, in both hESC and hiPSC, HHT treatment led to rapid and strong down-regulation of Mcl-1, an important anti-apoptotic member of the Bcl-2 family, as well

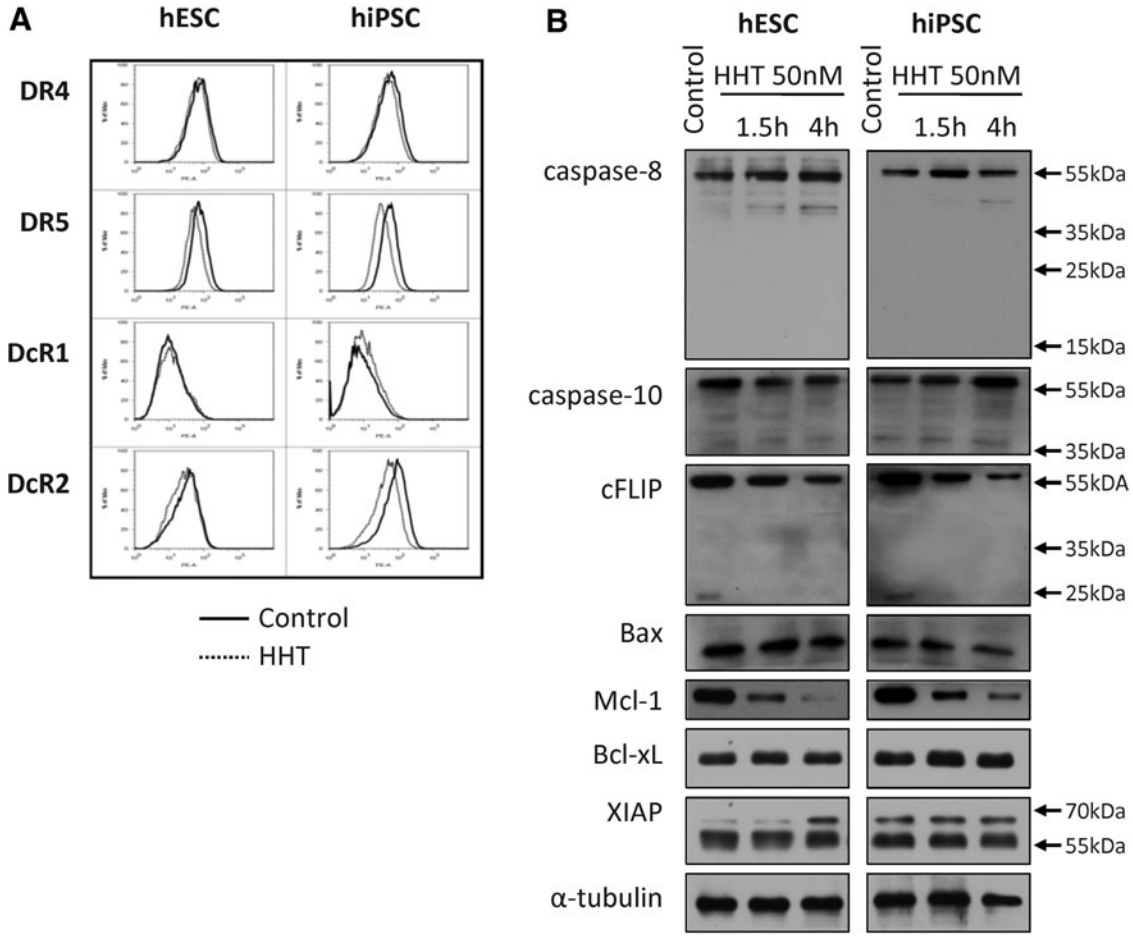

FIG. 4. Inhibition of protein synthesis in hESC and hiPSC rapidly reduces levels of endogenous cFLIP and Mcl-1. The hESC (CCTL 14) and hiPSC (clone 4) cells were left untreated (control) or treated with 50 nM HHT for 1.5 and 4 h. **(A)** The expression of TRAIL receptors in untreated (control, *solid line*) and HHT-treated (HHT, *dashed line*) hESC and hiPSC after 4 h of treatment was compared by flow cytometry. Representative histograms of three independent experiments are shown. **(B)** Western blot analysis of the expression of extrinsic apoptotic pathway components in control and HHT-treated cells (1.5 and 4 h). Alpha tubulin was used as a loading control.

as a less pronounced, but reproducible, suppression of cFLIP-L expression (Fig. 4B). Importantly, the potent short anti-apoptotic variant of cFLIP (cFLIP-S) was not detected after 1.5 h from the start of HHT treatment (Fig. 4B). It is of note that down-regulation of cFLIP and Mcl-1 was also achieved in cells exposed to CHX instead of HHT (Supplementary Fig. S3D).

cFLIP represents a safeguard protecting human pluripotent stem cells against TRAIL-induced apoptosis

The data obtained from sensitization experiments indicated that a small number of molecular players, namely cFLIP and Mcl-1, might underlie the resistance of hESC and hiPSC to activators of the external apoptotic pathway. To address the significance of cFLIP and Mcl-1 as regulatory elements, we generated recombinant lentiviruses expressing shRNA specific to each of these proteins and prepared clones of transduced hESC from the CCTL14 cell line. However, on selection with puromycin and subsequent analysis of target protein expression, we noticed that we could effectively down-regulate expression of cFLIP but not Mcl-1 (Fig. 5A and data not shown). Although we tested three different shRNAs targeting Mcl-1, the surviving cells showed either no change or only a very modest decrease in Mcl-1 protein expression.

Cells with down-regulated cFLIP expression (achieved using two different shRNA-expressing lentiviruses) and mock-transduced cells expressing nontargeting shRNA retained their basic stem cell phenotype and were used for all subsequent experiments. Initial experiments analyzed the behavior of the mock- and shFLIP-transduced cells after treatment with TRAIL by light microscopy. Untreated cells, TRAIL-treated, and mock-transduced cells appeared normal without any significant signs of cell death (Fig. 5B). In sharp contrast, cultures of hESC with knocked-down cFLIP (by either of the two shRNAs, only shFLIP1 is shown) typically showed massive cell detachment, clearly indicating execution of the apoptotic process (Fig. 5B). Consistent with the morphological observations, quantification of this phenomenon using an antibody against cleaved PARP revealed a significant increase in apoptotic cells from approximately 2%–3% in untreated cells to 25%–30% in TRAIL-treated cells with down-regulated expression of cFLIP (Fig. 5C).The

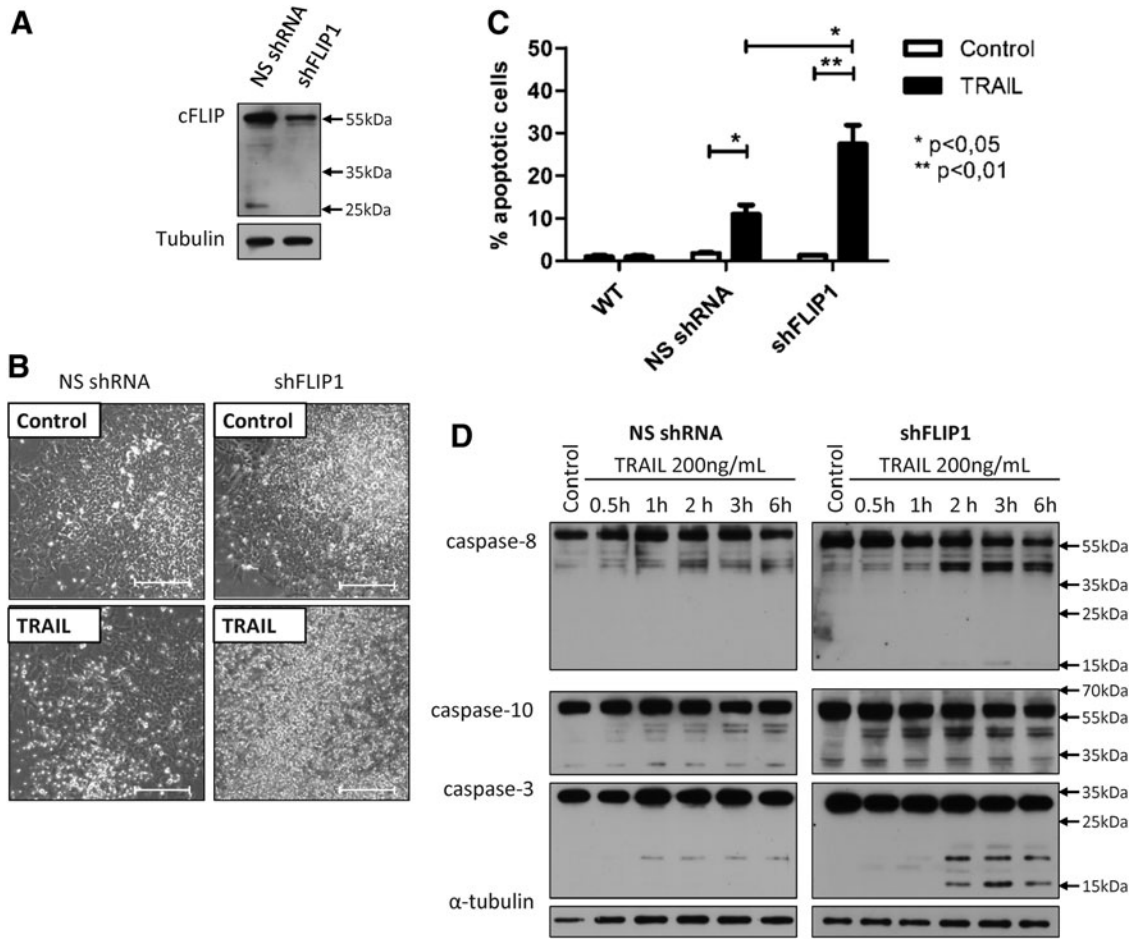

FIG. 5. Knock-down of cFLIP sensitizes hESC to TRAIL-induced apoptosis. Stable cell lines were established following transduction of the hESC line CCTL14 with a lentivirus expressing nontargeting, nonsilencing shRNA (NS shRNA) or shRNA targeting cFLIP mRNA (shFLIP1). **(A)** Western blot showing a decrease in cFLIP levels in shFLIP1-transduced cells compared with NS shRNA-transduced cells. Alpha tubulin was used as a loading control. **(B)** NS shRNA and shFLIP1 cells were left untreated (control) or treated with 200 ng/mL TRAIL. The morphology of colonies and cell detachment were observed by light microscopy after 6 h of treatment. **(C)** Nontransduced (WT), NS shRNA-transduced, and shFLIP1-transduced cells were left untreated (control) or treated with 200 ng/mL TRAIL for 6 h. Induction of apoptosis was determined as in Figure 3B. Each bar represents the mean of $n = 3$ experiments, error bars show ± SEM. **(D)** Western blot analysis of the activation of the extrinsic apoptotic pathway in NS shRNA and shFLIP1-transduced cells after 30 min and 1, 2, 3, and 6 h of treatment with 200 ng/mL TRAIL. Alpha tubulin was used as a loading control.

number of cells positive for cleaved PARP also increased by approximately 10% in TRAIL-treated mock-transduced cells. In TRAIL-treated cells with down-regulated expression of cFLIP, but not in mock-transduced cells, we also observed pronounced cleavage of initiator caspase-8 and -10, as well as effector caspase-3 (Fig. 5D).

To further investigate the functionality of such caspase cleavage, we exposed these cells to a combination of TRAIL with pan caspase inhibitor Z-VAD-FMK and caspase-8 inhibitor Z-IETD-FMK, respectively. As shown in Supplementary Fig. S5, both inhibitors significantly reduced apoptosis and cell death, in accordance with reduced activation of initiator caspase-8 and effector caspase-3. Recently, cFLIP was found to also prevent cells from undergoing necroptosis [49]. To test the possibility that necroptosis may contribute to the pool of dying cells in TRAIL-treated shFLIP cells, the necroptotic pathway was inhibited by necrostatin-1. In this experiment, no change in cell death was observed (Supplementary Fig. S5A, B).

Discussion

hESCs and induced pluripotent stem cells are highly vulnerable to various adverse situations and respond to such stresses by activation of the intrinsic apoptotic pathway [6,50]. However, virtually nothing is known about the other main branch of canonical apoptotic signaling—induction of extrinsic apoptosis via activated death receptors in the TNFR family. In this communication, we describe for the first time the expression of the major death domain-containing receptors, TNFR1 and Fas/CD95, and the functional state of TRAIL receptors, in ICM-derived hESC and hiPSC obtained by fibroblast reprogramming. We found that Fas/CD95 expression is virtually undetectable in either cell type (Fig. 1A,

B) but increases dramatically (by more than an order of magnitude) during differentiation of hESC into neural progenitors (unpublished data). The expression of TNFR1 on human pluripotent stem cells is also rather low, especially when compared with the TRAIL receptors DR4 and DR5. Analysis of the expression and function of these two death receptors in mouse embryonic stem cells (mESCs), however, provides contradictory results. While earlier reports argue for functional Fas/CD95 and TNFR expression on mESCs [51–53], more recent publications document a lack, or very low expression, of either Fas/CD95 or p55 TNFR in these cells [54,55], in agreement with our data for hESC and hiPSC, at least regarding the Fas/CD95 receptor. Notably, in their study, Kim and colleagues reported that Fas/CD95 expression is induced by environmental cell stress-inducing pollutants [56]. In contrast to these death receptors, the pro-apoptotic TRAIL receptors DR4 and DR5 are highly expressed in both types of human pluripotent stem cells evaluated in our study, and their expression levels are similar to those measured on adult human fibroblasts and hESC-derived neural progenitors (unpublished data). Interestingly, mouse blastomeres and trophectoderm cells were shown to express a death domain-containing TRAIL receptor and its ligand, supporting our findings for ICM-derived hESC and engineered hiPSC [57].

The expression of death receptors is a necessary, but not sufficient, prerequisite for full execution of the extrinsic apoptotic pathway. Despite the expression of both pro-apoptotic TRAIL receptors and all other proteins that are necessary for the efficient transduction of TRAIL-induced apoptotic signaling (see Fig. 1C), human pluripotent stem cells were resistant to TRAIL-induced apoptosis, similar to mESCs [57]. This resistance can be imposed by a number of signaling pathways, but, in principle, these mechanisms cluster into two regulatory nodes—a proximal one related to DISC-mediated activation of the initiator caspase-8 and a distal one relying on mitochondrial and postmitochondrial regulation of apoptotic signaling [39,58]. One of the essential regulatory proteins at the DISC node is cFLIP, which can competitively block caspase-8 self-processing. In particular, its shorter splice variants, cFLIP-R and -S, are very effective inhibitors [59,60]. In hESC and hiPSC, cFLIP-S, cFLIP-L (although to a lesser extent), and the anti-apoptotic Bcl-2 family protein Mcl-1 rapidly responded to HHT-mediated sensitization to TRAIL-induced apoptosis. We were able to confirm the importance of the first node's regulatory role by shRNA-mediated cFLIP-L/S knock-down in hESC, which significantly and strongly sensitized the cells to TRAIL-induced apoptosis.

The question remains as to why mouse and, as we document, also human pluripotent stem cells would need to express activatable TRAIL receptors. In mice, gene targeting of *TNFRSF10B* (coding for the only pro-apoptotic TRAIL receptor DR5/Killer) or its ligand does not hamper embryonic development, although in adult mice it can accelerate both spontaneous and chemically induced tumorigenesis [61,62]. However, in the context of recent knowledge on the relatively high sensitivity of human pluripotent stem cells to DNA damage and/or virus-induced apoptosis, our findings are not so surprising [6,57,63]. Recent studies document that hESC are, in general, equipped to undergo fast apoptosis because of elevated levels of several BH3-only pro-apoptotic Bcl-2 family members and the presence of active Bax at the Golgi that can rapidly translocate to mitochondria [8,9]. Importantly, DR4 was recently shown to be instrumental for activation of poised Bax after DNA damage [64], and TRAIL and its receptors are among the death ligands/receptors that are generally considered stress sensors. From this perspective, the expression of functional TRAIL receptors in addition to preactivated Bax and higher expression of the pro-apoptotic BH3-only protein could represent another level of protection of human pluripotent stem cells against malicious and potentially genotoxic extracellular stresses such as radiation or chemicals.

Acknowledgments

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Author Disclosure Statement

The authors state that no competing financial interests exist.

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Address correspondence to:
Dr. Ales Hampl
Department of Histology and Embryology
Faculty of Medicine
Masaryk University
Kamenice 126/3
Brno 625 00
Czech Republic

E-mail: ahampl@med.muni.cz

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