Acquired immune evasion is one of the mechanisms that contributes to the dismal prognosis of cancer. Recently, we observed that different $\gamma\delta$ T cell subsets as well as CD8$^+$ $\alpha\beta$ T cells infiltrate the pancreatic tissue. Interestingly, the abundance of $\gamma\delta$ T cells was reported to have a positive prognostic impact on survival of cancer patients. Since $\gamma\delta$ T cells utilize TNF-related apoptosis inducing ligand (TRAIL) for killing of tumor cells in addition to granzyme B and perforin, we investigated the role of the TRAIL-/TRAIL-R system in $\gamma\delta$ T cell-cytotoxicity toward pancreatic ductal adenocarcinoma (PDAC) and other cancer cells. Coculture of the different cancer cells with $\gamma\delta$ T cells resulted in a moderate lysis of tumor cells. The lysis of PDAC Colo357 cells was independent of TRAIL as it was not inhibited by the addition of neutralizing anti-TRAIL antibodies or TRAIL-R2-Fc fusion protein. In accordance, knockdown (KD) of death receptors TRAIL-R1 or TRAIL-R2 in Colo357 cells had no effect on $\gamma\delta$ T cell-mediated cytotoxicity. However, KD of decoy receptor TRAIL-R4, which robustly enhanced TRAIL-induced apoptosis, interestingly, almost completely abolished the $\gamma\delta$ T cell-mediated lysis of these tumor cells. This effect was associated with a reduced secretion of granzyme B by $\gamma\delta$ T cells and enhanced PGF2 production as a result of increased expression level of synthetase cyclooxygenase (COX)-2 by TRAIL-R4-KD cells. In contrast, knockin of TRAIL-R4 decreased COX-2 expression. Importantly, reduced release of granzyme B by $\gamma\delta$ T cells cocultured with TRAIL-R4-KD cells was partially reverted by bispecific antibody [HER2xCD3] and led in consequence to enhanced lysis of tumor cells. Likewise, inhibition of COX-1 and/or COX-2 partially enhanced $\gamma\delta$ T cell-mediated cytotoxicity. The combination of bispecific antibody and COX-inhibitor completely restored the lysis of TRAIL-R4-KD cells by $\gamma\delta$ T cells. In conclusion, we uncovered an unexpected novel role of TRAIL-R4 in tumor cells. In contrast to its known pro-tumoral, anti-apoptotic function, TRAIL-R4 augments the anti-tumoral cytotoxic activity of $\gamma\delta$ T cells.

Keywords: TRAIL-receptor 4, $\gamma\delta$ T cell-cytotoxicity, pancreatic cancer, COX (cyclooxygenase), PGF2, granzyme B, TRAIL, bispecific Ab
INTRODUCTION

The prominent role of death ligands Tumor necrosis factor (TNF)-α, TNF-related apoptosis inducing ligand (TRAIL) and CD95-L in tumor biology is undoubted, yet not fully understood. Particularly, the TRAIL/TRAIL-R system remains still widely unexplored, due to its complexity. There are four plasma membrane expressed TRAIL receptors, TRAIL-R1-R4 and one soluble TRAIL receptor, Osteoprotegerin (1–6). In addition to binding of TRAIL, all plasma membrane receptors can interact with each other forming homo- and hetero-complexes and this can take place prior to and/or following TRAIL-binding. Two of the receptors, TRAIL-R1 and TRAIL-R2 carry the so-called death domain and are therefore able to induce cell death. This function of TRAIL-R1/-R2 is believed to be important for the immune surveillance of tumors. Accordingly, down regulation of TRAIL-R1 and/or TRAIL-R2 at the cell surface are characteristic features of cancer cells leading to tumor escape and acquired resistance of tumor cells toward TRAIL-induced apoptosis (7, 8). In addition, to inducing cell death, TRAIL-R1/-R2 are able to trigger several non-apoptotic signaling pathways which may increase tumor malignancy by influencing the tumor cells themselves as well as the tumor microenvironment via secreted inflammatory cytokines (1, 9–12). Two additional TRAIL receptors, TRAIL-R3 and TRAIL-R4, do not contain a functional death domain and therefore unable to induce cell death. Instead, these receptors negatively regulate TRAIL-induced apoptosis via binding of TRAIL and/or interaction with TRAIL-R1/-R2 (2–4, 13, 14). Of note, through their role as decoy receptors, TRAIL-R3, and TRAIL-R4 can act either autonomously at the cell or at a supracellular level thus influencing the sensitivity of other cells in the tumor microenvironment to TRAIL-induced apoptosis (15).

Importantly, TRAIL death receptors are frequently overexpressed in tumors but instead of being present at the plasma membrane they are mainly localized intracellularly, in the cytoplasm and in the nucleus. Emerging evidence suggests that these intracellular TRAIL receptors exert functions different from that of the plasma membrane expressed receptors and can influence or contribute to the malignant progression (7, 16–22). Accordingly, it has been shown for some tumor entities that high intracellular expression of mainly TRAIL-R2, also TRAIL-R1, correlates with poor patients' prognosis (7).

Only recently, specific functions of intracellular TRAIL receptors were uncovered. Thereof, nuclear TRAIL-R2 expressions were observed in human pancreatic cancer cell lines (19). In addition, cytoplasmic TRAIL-R1 and TRAIL-R2 have been shown to induce apoptosis in response to the unresolved unfolded protein response (23, 24).

In contrast to the plethora of data dealing with TRAIL-R1/-R2 functions in tumor cells, the function of TRAIL-R4 is largely unexplored and is almost exclusively deduced from overexpression studies. Unlike TRAIL-R3, which is only anchored in the plasma membrane and is therefore unable to induce intracellular signaling, TRAIL-R4 contains a complex intracellular part which is homologous to those of TRAIL-R1 and TRAIL-R2 but its death domain is truncated and therefore non-functional (2, 4). Consistently, several reports showed that beside the ability to inhibit TRAIL-mediated apoptosis TRAIL-R4 can induce non-apoptotic signaling pathways like NF-κB and AKT, which might contribute to its pro-tumoral function (2, 25). This might explain the reported overexpression of TRAIL-R4 in tumors, which in some cases could be correlated to a more malignant phenotype and poor patients’ prognosis (17, 26, 27). On the other hand, some tumors down regulate the expression of TRAIL-R4, suggesting a context-dependent and/or cancer specific function of this receptor (22, 28, 29).

Tumors and the tumor microenvironment contain a network of immunoregulatory and immunosuppressive mediators which influence effector functions of tumor-infiltrating cells including γδ T cells. γδ T cells play a major role in immune surveillance by recognizing overproduced metabolites and stress-induced surface molecules on cancer cells. Aside from the classical cytotoxic mediators (e.g., granzymes), death ligands TNF, CD95, and TRAIL are used by the different γδ T cell subsets to lyse a broad range of tumor cells (30). γδ T cells represent a numerically small population of T cells in the peripheral blood with a predominance of Vγ9Vδ2 T cell receptor (TCR)-expressing T cells. Vγ9Vδ2 T cells recognize pyrophosphates of the isoprenoid pathway of prokaryotes or dysregulated mevalonate pathway of tumor cells in a butyrophilin 3A (BTN3A)-dependent manner (31, 32). The Vδ1 γδ T cells are rare in the peripheral blood of healthy donors but are frequently found in cancer patients (30, 33–35). Vδ1 T cells express one of various Vγ chains (Vγ 2, 3, 4, 5, or 8) and recognize microbial- and self-lipids bound to CD1d molecules or stress-induced MHC class-I related chain A (MICA), that are frequently expressed on tumor cells including pancreatic ductal adenocarcinomas cells (PDAC) (36–38). Both γδ T cell subsets highly express NKG2D, the activating receptor for MICA and MICB and lyse different tumor cells in a NKG2D-dependent manner (39). Shedding of NKG2D ligands from PDAC cells is reported as one of the tumor escape mechanism (36). Beside the interaction of NKG2D with MICA or MICB, the interaction with other adhesion- and costimulatory molecules such as CD54, CD80/86, CD154, and their corresponding ligands CD11a/CD18, CD28, and CD40 expressed on γδ T cells are conducive for triggering their effector function (40, 41). Both γδ T cell subsets recognize their antigens in a HLA-independent manner and infiltrate tumors which is the underlying principle of γδ T cell-based immunotherapies (42–45). The abundance of intratumoral γδ T cells was identified as one of the most important prognostic factor associated with a positive outcome in a recent meta-analysis (46). While they exert pronounced antitumor efficacy and therefore are potential candidates for immunotherapy, Vδ2- as well as Vδ1 γδ T cells can also be inhibited by

Abbreviations: bsAb, bispecific antibody; COX, cyclooxygenase; mAb, monoclonal antibody; MICA, MHC class-I related chain A; PDAC, pancreatic ductal adenocarcinoma; PG, prostaglandin; RTCA, Real Time Cell Analyzer; TCR, T cell receptor TRAIL, TNF-related apoptosis inducing ligand; PBMC, peripheral blood mononuclear cells.
immunosuppressive cells and mediators such as cytokines in the tumor-microenvironment as well as by different inherent tumor escape mechanisms (47). Recently, we observed that the majority of PDAC cells are highly resistant to Vδ2 T cell-mediated lysis, which can be explained by different mechanisms ([48] and unpublished observations). In this context, we demonstrated that the coculture of PDAC Colo357 and other tumor cells such as breast cancer, MDA-MB-231, and cervical cancer HeLa cell lines with Vδ2 T cells increased the prostaglandin (PG) synthetase cyclooxygenase (COX)-2 expression in tumor cells and thereby the release of PGE2, which in turn significantly inhibited Vδ2 T cell-mediated cytotoxicity (48). Similar to Vδ2 T cells, Vδ1 T cells infiltrate tumors and their activity could be regulated by different mechanisms preventing their effective anti-tumor response. Since Vδ T cells may use TRAIL to kill tumor cells, we investigated the role of TRAIL/TRAIL-R system with a focus on TRAIL-R4 on the sensitivity of tumor cells toward Vδ T cell-induced cytotoxicity.

MATERIALS AND METHODS

Establishment of Vδ T Cell Lines
Vδ T cell lines were established from peripheral blood mononuclear cells (PBMC) of healthy adult donors (#1 and #2 in Figures 2A, 3; additional donors in Figures 2B, 7, 8) and cancer patients (#3 in Figures 2, 3; additional donors in Figures 2B, 7, 8). PBMC were isolated from leukocyte concentrates obtained from healthy blood donors and provided by the Department of Transfusion Medicine of the University Hospital Schleswig-Holstein (UKSH) in Kiel, Germany. In addition, heparinized blood from cancer patients was obtained from the Department of General Surgery of the municipal hospital. In accordance with the Declaration of Helsinki, written informed consent was obtained from all donors, and the research was approved by the relevant institutional review boards (ethic committee of the Medical Faculty of the CAU Kiel, code number: D445/18).

PBMC were isolated by Ficoll-Hypaque (Biochrom, Berlin, Germany) density gradient centrifugation and cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 25 mM HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin, 10% fetal bovine serum (FBS) [complete medium]. To expand Vδ1-expressing Vδ T cells, 24-well plates were coated with 100 µL of 0.5 µg/mL anti-Vδ1 TCR monoclonal antibody (mAb) clone R9.12 (Beckman Coulter, Krefeld, Germany) overnight at 4°C. After washing the wells, 106 PBMC/well were cultured with a final concentration of 1 µg/mL anti-CD28 mAb clone CD28.2 (Biolegend, Fell, Germany) and 50 U/mL IL-2 (Novartis, Basel, CH) for 14 to 21 days. Since resting, initially stimulated Vδ1 Vδ T cells produced only low amounts of IL-2, 50 U/mL IL-2 were added every other day. After 2–3 weeks, Vδ1-expressing Vδ T cell lines had a purity >40–60% and were labeled with anti-TCRB mAb clone IP26 (Biolegend) and subjected to magnetic separation in order to deplete remaining αβ T cells.

Additional large-scale expansion of Vδ1 T cells (that continued to coexpress various Vγ-chains) was performed in rIL-2-supplemented medium with repetitive restimulation using 0.5 µg/mL phytohaemagglutinin (Thermo Fisher Scientific, Langenbold, Germany) and irradiated PBMC (20 × 106 cells) and/or EBV-transformed B cell lines (2 × 106 cells) as feeder cells for 20 × 106 cells Vδ1 T cells. Dead feeder cells were removed 3–4 days after restimulation by Ficoll-Hypaque density gradients. Purity of Vδ1 γδ T cells was >98% as analyzed by flow cytometry.

Tumor Cell Lines, Establishment of Clones, and Cell Culture Conditions
Human PDAC cell lines PancTuI and Colo357 as well as breast cancer cell line MDA-MB-231 were cultured in RPMI 1640 medium with 2 mM glutamine, 1 mM sodium pyruvate (all from Gibco, Darmstadt, Germany) and 10% FBS (PAN Biotech, Aidenbach, Germany) under regular conditions (5% CO₂, humidified, 37°C). In addition, cervical cancer cell line, HeLa cells, with TRAIL-R4 overexpression and the appropriate control cells were established and gratefully provided by Prof. Olivier Micheau (INSERM, Dijon, France). HeLa cells were cultured in DMEM with 4.5% D-Glucose with 10% FBS (PAN Biotech), 2 mM Glutamine and 1 mM sodium pyruvate and 2.5 µg/mL puromycin for selection under regular conditions.

For stable knockdown of TRAIL-R4, cells were transfected with the GIPZ Lentiviral Human TNFRSF10D shRNA or with the non-silencing control [cloneIDs: V2LHS_16774 (led to the establishment of clone (a), V2LHS_16773, V3LHS_344449 led to the establishment of clone (b), V3LHS_344451; Dharmaco, GE Healthcare, Lafayette, CO, USA] and selected with puromycin (Colo357/MDA-MB-231 cells with 2 µg/mL and PancTuI cells with 1 µg/mL). Additionally, the same method was used to knockdown TRAIL-R1 (cloneID: V3LHS_383714) and TRAIL-R2 (cloneID: V2LHS_16711). To inhibit cell signaling pathways, cells were seeded in 6-wells (5 × 10⁶/well) and 24 h later treated for 8 h with 2 µM Insolution™ MG-132 proteasome inhibitor (#474791, EMD Millipore Corp., USA), 10 µM MEK inhibitor U0126 (Promega, Madison, USA) or 1 µM MK-2206 2HCL AKT-1, -2, -3 inhibitor (#S1078, Selleckchem, USA).

Absence of mycoplasma was routinely confirmed by RT-PCR (Venor® GEM classic, Minerva Biolabs GmbH, Germany).

RNA Interference
8.5 × 10⁶ HeLa cells were seeded in 15 cm Petri dish, and incubated for 24 h under normal conditions. The cells were transfected with 10 µM TRAIL-R4 siRNA or non-targeting control pool, on-Targetplus, human siRNA, smartpool (TNFRSF10D; L-008092-01-0005, non-targeting; D-001810-10-20, both from Dharmaco/Horizon Discovery, USA) using Lipofectamine® RNAiMAX reagent (ThermoFisher Scientific, USA) according to manufacturer’s Protocol.

Real-Time Polymerase Chain Reaction
Cells were homogenized with QIAshredder (QUIAGEN, Hilden, Germany) and total RNA was isolated with a RNeasy Mini Kit (QUIAGEN). cDNA synthesis was performed using Maxima first strand cDNA synthesis kit (K1671, Thermo Fisher Scientific, USA). The expression of TRAIL-R4 was determined by qPCR using TaqMan assays (Thermo Fisher Scientific, USA) and a 7900HT Fast qPCR system (Thermo Fisher Scientific, USA). The
expression levels were calculated relative to the expression of the housekeeping gene TATA-binding protein (TBP) by ΔΔCT method. Primers were purchased from Thermo Fisher Scientific (TBP (Hs00427620_ml) and TRAIL-R4 (Hs00388742_ml)).

Flow Cytometry

For the analysis of the purity of the V61 γδ T cells, cells were stained with the following mAb: anti-CD3 (clone SK7, BD Biosciences, Heidelberg, Germany), anti-TCRγδ (clone 11F2, Miltenyi Biotech, Bergisch Gladbach, Germany), anti-TCRβ (clone IP26, Biologend), anti-TCRVδ1 (clone TS8.2, Thermo Fisher Scientific), anti-TCRVγ9 [clone 7A5, (49)], anti-TCRVγ2, -3 or -4 [clone 23D12, (50)], anti-TCRVγ8 [clone R4.5.1 (51)] or corresponding isotype controls (BD Biosciences or Biologend).

For surface staining of adhesion-and costimulatory molecules and TNF-receptor family members, 2 × 10^5 cells were washed and stained with mAb as follows: anti-CD44 (clone HCAM, BD Biosciences), anti-CD54 (clone 84H10, Beckman Coulter), anti-CD80 (clone L307.4, BD Biosciences), anti-CD86 (clone 37301, R&D Systems, Wiesbaden, Germany), anti-CD154 (clone 24–31, Ancell), anti-MICA (clone 159227, R&D Systems) and anti-MICB (clone 236511, R&D Systems) or appropriate isotype controls for 25 min. After two washing steps, cells were measured by flow cytometry.

For intracellular staining, 2 × 10^5 tumor cells were treated (or not) with 1 μg/mL bispecific antibody (bsAb) [HER2xCD3] overnight, washed, permeabilized and fixed with Cytofix/Cytoperm kit (BD Biosciences) and stained with anti-COX-1-FITC/anti-COX-2-PE mAb (clone AS70/AS67, BD Biosciences) or with unconjugated COX-1 (clone Cox111, Thermo Fisher Scientific) followed by second-step with PE labeled goat-anti-mouse (Thermos Fisher Scientific) or the appropriate isotype controls following the procedures outlined by the manufacturer. After washing, all samples were analyzed on a LSR Fortessa flow cytometer (BD Biosciences) using DIVA 8.0 software.

For TRAIL-R cell surface expression analysis, one million cells were directly stained with APC-labeled mouse mAb (all from R&D Systems, biotechne) as follows: IgG1 isotype control (clone #11711) or anti-TRAIL-R1/TNFRSF10A (clone #69036), anti-TRAIL-R3/TNFRSF10C (clone #90906), and TRAIL-R4/TNFRSF10D (clone #104918) as well as with IgG2b isotype control (clone #71908) or anti-TRAIL-R2/TNFRSF10B (clone #71908) and analyzed on a FACS Calibur using Cell-Quest Software.

Western Blot Analysis

Cells were lysed in RIPA buffer supplemented with Complete Protease Inhibitor Cocktail and PhosphoStop (both from Roche, Mannheim, Germany) and Western blot analyses were performed as described previously (10). Primary antibodies were purchased from: Cell Signaling, Frankfurt, Germany [anti-ERK1/2 (9102), anti-phospho-ERK1/2 (9106), anti-TRAIL-R4 (8049), anti-TRAIL-R2 (3696), anti-mouse-IgG-HRP (7076), anti-rabbit-IgG-HRP (7074)]; EMD Millipore Corp., USA [TRAIL-R1 (AB16955)], BD Biosciences, USA [anti-COX-2 (610203)], TRAIL-R3 (DcR1, im-245-1, Imgenex, San Diego, USA), HSP90α/β (Santa Cruz, sc-7947), and from Sigma-Aldrich [anti-β-actin (A5441)].

Real Time Cell Analyzer

Cytotoxicity against adherent cancer cells was measured by a Real Time Cell Analyzer (RTCA, X-CelLibge, ACEA, San Diego, CA, USA) in triplicates as described elsewhere (34, 44, 52, 53). To monitor the impedance of the cells via electronic sensors on the bottom of 96-well micro-E-plate every 5 min for up to 23–32 h, 10^6 tumor cells/well in complete medium were added to the plates. After 23–32 h, medium with or without previously titrated optimal concentrations of construct or substances were added. One μg/mL (final concentration) bsAb [HER2xCD3] or corresponding control constructs as well as 50 μM COX-1/2 inhibitor Indomethacin, 30 μM selective COX-2 inhibitor DuP697 (both from Tocris Bioscience, Bristol, UK), 2.5 mM selective COX-1 inhibitor valeryl salicylate (Cayman Chemical Company, MI, USA), 20 μM pan-caspase inhibitor zVAD-fmk (Bachem, Bubendorf, Switzerland) or 1 μg/mL PGE2 (Tocris Biosciences, Bristol, UK) were added in additional experiments 1 h before addition of γδ T cells. The bsAb [HER2xCD3] targets CD3-expressing T cells to HER2 + tumor cells (34). Impedance of the cells reflects changes in cellular parameters such as morphological changes (e.g., adherence, spreading), cell proliferation and cell death and is expressed as an arbitrary unit called cell index (CI). Since the initial adherence in different wells can differ slightly, the CI was normalized to 1 after the cells have reached the linear growth phase. After 23–32 h, V81 and V82 T cell lines with a previously titrated effector/target (E/T) ratio of 25:1 together with 12.5 U/mL IL-2 were added to the tumor cells. Loss of impedance of tumor cells is shown as decrease of the normalized CI due to V81 T cell lines-induced lysis of the malignant cells. Different cancer cells were treated with 1% Triton X-100 (final concentration) as a positive control for killing. For the precise analysis of cytotoxicity, the cells were monitored every minute for the indicated time points. By using the RTCA software (version 2.0.0.1301 Copyright © 2004–2012 ACEA Biosciences Inc.) the raw data files were exported to Microsoft Excel [version 14.0.7128.5000 (32-bit)] for further calculation and described as follows. The mean of Triton-X-100 samples was calculated and defined as 100% lysis over 10 h after addition of T cells. The ratio of each sample (control- or TRAIL-R4-KD cells plus V81 or V82 T cells) to its appropriate control (identical control- or TRAIL-R4-KD without V81 or V82 T cells) was calculated and the ratio was normalized to maximal inducible lysis by Triton-X-100. In some experiments, percentage of lysis was calculated at 4 h after addition of T cells (Figures 2B, 7C).

In other experiments, where an increase of impedance of tumor cells instead of a decrease in the presence of effector cells is shown (due to resistance against lysis), fold change in CI was calculated at 4 h after addition of T cells (Figures 3B, 8C). Time point zero was defined as first measurement after addition of T cells.

Viability Assay

Fifteen thousand cells were seeded/96-well and incubated for 24 h. The cells were treated with 50 ng/mL recombinant
human sTRAIL/Apo2L (#310-04, PeproTech, Germany) for 24 h. Cellular viability was detected using EZ4U (#BI-5000, Biomedica Medizinprodukte GmbH, Vienna) according to manufacturer's protocol.

Enzyme-Linked Immunosorbent Assay

Ten thousand PDAC cells transfected with TRAIL-R4-specific shRNA (clone a and b) or scrambled shRNA (control) were seeded in 96-well flat bottom microtiter plates (Nunc,

![Western Blot Analysis](image1)

![RT-PCR](image2)

![Flow Cytometric Analysis](image3)

![Viability Assay](image4)

**FIGURE 1** | Characterization of control- and TRAIL-R4 knockdown Colo357 cells. (A) Colo357 cells were transduced with either control lentiviral shRNA or TRAIL-R4 specific shRNA to stably knockdown TRAIL-R4. The protein level of TRAIL-R4 was detected in the whole cell lysate of the transduced cells via western blot. Additionally the mRNA level of TRAIL-R4 in TRAIL-R4 KD clones [TR4 KD (a) and (b)] was measured via qPCR (B), shown is the mean of three biological replicates. (C) Furthermore, cell surface expression of TRAIL-R4 in the TRAIL-R4-KD clones (a) and (b) [gray filled histograms; thin lines, isotype control] was detected by using flow cytometry. Shown is a representative histogram out of six biological replicates and an overlay of control- (gray filled histograms) vs. TR4-KD Colo357 cells (bold lines). (D) The viability of cells under TRAIL treatment (50 ng/mL) was determined by EZ4U assay. Shown is the mean of three biological replicates. Student t-test was done to determine significance (* $< 0.05$ p-value). (E) Ten thousand control- (light green line) or TRAIL-R4-knockdown (TR4-KD) Colo357 cells [TR4-KD (a), dark blue line; TR4-KD (b), orange line] were cultured in complete medium for 95 h on E-plates. The bottom of the E-plates were covered with electronic sensors that measured the impedance of these adherent tumor cells expressed as an arbitrary unit called cell index (CI) every 5 min. The arrows mark the addition of substances after 26 h as follows: medium [light green line for control KD Colo357 cells, dark blue line for TR4-KD (a), orange line for TR4-KD (b) Colo357 cells], 100 ng/mL recombinant TRAIL [dark green line for control KD cells, red line for TR4-KD (a), and light blue line for TR 4 KD (b) cells] or Triton-X-100 to induce maximal lysis [black line]. CI was then measured for additional 69 h. The loss of tumor cell impedance and thus a decrease of CI correlated with lysis of tumor cells. The average of triplicates and standard deviation were calculated; one representative experiment out of three is shown.
Wiesbaden, Germany). After 23 h, 1 µg/mL (final concentration) bsAb [HER2xCD3] or 50 µM Indomethacin or the combination of both were added 1 h before addition of Vδ1 T cell lines (E/T ratio: 25:1) supplemented with 12.5 U/mL rIL-2 for further 24 h. To quantify PGE2 released by PDAC cells alone or after coculture with Vδ1 T cell lines as well as granzyme B released...
FIGURE 3 | Neutralization of TRAIL does not reverse the inhibitory effects of the TRAIL-R4 KD in Colo357 cells on Vδ1 T cell-mediated cytotoxicity. (A) Ten thousand control KD—or TRAIL-R4 KD [TR4 KD (a) or TR4 KD (b)] Colo357 cells per well were cultured in complete medium overnight. Cell Index (CI) was analyzed in 5 min steps over ~32 h. After overnight adherence, Colo357 cells were cultured with additional complete medium [control: green, TR4 KD (a): blue dark or TR4 KD (b): orange line] or positive control Triton-X-100 (black line). After 32 h, Colo357 cells were cocultured with two Vδ1 γδ T cell lines of different donors (#2, red lines and #3, purple lines) with an E/T ratio of 25:1 and 12.5 IU/mL rIL-2 in the presence of medium (red or purple lines) or 1 µg/mL TRAIL mAb (dark green lines). Lysis of tumor cells was measured after normalization to 1 in one min steps for >18 h as indicated. The average of three replicates with SD is represented for each tumor cell line with effector cells of one representative healthy donor (#2) and one pancreatic cancer patient (#3) in independent experiments.

(B) The culture conditions were similar to the ones described in (A) with the difference that only control KD—or TRAIL-R4 KD [TR4 KD (a)] Colo357 cells were applied as target cells. After 32 h, Colo357 cells were cocultured with five different Vδ1 γδ T cell lines of different donors with an E/T ratio of 25:1 and 12.5 IU/mL rIL-2 in the presence of medium or 20 µM zVAD-fmk. Each symbol represents a different donor. Black bars represent mean of the five independent experiments. Cytotoxicity was analyzed by Real-Time Cell Analyzer and fold change in Cell Index (CI) was calculated using formula as follows: CI – Fold change = 1 – (1 – (S/C)) * (1 – M); S, CI value of the sample; C, value of the medium control; M, Mean of CI value of Triton-X-100 sample.
by Vδ1 T cell lines cocultured with PDAC cells in the absence or presence of bsAb or COX1/2 inhibitor, supernatants were collected after incubation time and stored at −20°C until use. PGE2 was measured by Prostaglandin E2 Parameter Assay Kit (#SKGE004B) and human granzyme B by a sensitive sandwich ELISA (both from R&D System) in duplicates following the procedures outlined by the manufacturer.

Statistics
Statistical analysis was assessed by One-way-Anova or Wilcoxon rank sum test using Graph Pad Prism (Graph Pad Software, Inc., La Jolla, CA, USA) or by a para-metrically t-test using (Microsoft Excel). The level of significance of all statistical tests was set at 5%.

RESULTS
Knockdown of TRAIL-R4 Reduces Sensitivity of Cancer Cells to γδ T Cell-Induced Cytotoxicity
Although γδ T cells lyse a broad range of different tumor cells, they are largely ineffective against several other tumor cells. Yet, the mechanisms behind this deficit are not completely

![Figure 4](image-url)
Tawfik et al. TRAIL-Receptor 4 Modulates γδ T Cell-Cytotoxicity

understood. Beside granzyme B and perforin, γδ T cells can also utilize death receptor ligands, among them TRAIL, to mediate cytotoxicity against tumor cells (54, 55). TRAIL-R4 has been shown to be significantly up-regulated in PDAC tissue compared to non-malignant ducts from PDAC patients or non-cancer patients (27). Importantly, this receptor may act as a negative regulator of TRAIL receptor-mediated apoptosis in different tumor cells. Since our unpublished data showed the expression of TRAIL-R4 in different tumor cells, we wondered whether this receptor could at least be partially responsible for the establishment of a resistant phenotype of these cells to γδ T cell-induced cytotoxicity as we recently reported (48, 56).

To address this issue, we first generated Colo357 and MDA-MB-231 cells with suppressed expression of TRAIL-R4 by stable transfection with TRAIL-R4-specific shRNA. All resulting TRAIL-R4-knockdown (KD) cell lines expressed lower levels of TRAIL-R4 than the corresponding control cells transfected with non-silencing shRNA (Figure 1A, Supplementary Figures 1A,B). Two of these obtained Colo357 KD cells and one of the MDA-MB-231 KD cells, which showed the most efficient knockdown of TRAIL-R4 [Figure 1A; TRAIL-R4-KD lines (a) and (b), Supplementary Figures 1A,B], were selected for further investigations. In all TRAIL-R4-KD cells, the expression of the other TRAIL-receptors was comparable (Figure 1B, Supplementary Figures 1A,B). Beside the clear decrease of the total cellular content of TRAIL-R4 protein (Figure 1A, Supplementary Figures 1A,B) and TRAIL-R4-mRNA (Figure 1B), these clones also showed, albeit less pronounced, diminished levels of this receptor at the cell surface (Figure 1C, data not shown).

For functional validation of the TRAIL-R4-KD cells, their sensitivity to TRAIL-mediated cell death was determined by two different methods, an EZ4U-Assay, which measures the mitochondrial metabolic activity (Figure 1D) and the Real Time Cell Analyzer (RTCA) system, which measures changes in cell adherence using the impedance technology (Figure 1E). Consistent with its known inhibitory function, knockdown of TRAIL-R4 clearly increased the sensitivity of cells to TRAIL-mediated apoptosis (Figures 1D,E).

Next, we examined whether these cells are also more sensitive to γδ T cell-mediated cytotoxicity. For this purpose, we incubated the TRAIL-R4-KD cells or the control cells with different γδ T cell lines of different healthy donors and cancer patients and analyzed the lysis of tumor cells using RTCA system (Figure 2, Supplementary Figure 2). Here, in addition to Vδ2 γδ T cells, we also tested the killing capacity of Vδ1 γδ T cells, the cell population which we found to be mainly present in the tumor tissue (unpublished observation). After Colo357 cells were allowed to adhere for 24 h, the effector cells (>95% purity) were added and changes in Cell Index (CI) were monitored over 20 h.

RTCA analysis revealed that Colo357 cells were highly resistant to Vδ2 γδ T cell-induced cytotoxicity and less resistant but still not completely lysed by Vδ1 γδ T cells (Figure 2A). Surprisingly, knockdown of TRAIL-R4, instead of sensitizing the cells to γδ T cell-induced lysis, rendered these cells even more resistant. The acquired resistance of TRAIL-R4-KD cells toward γδ T cell-mediated cytotoxicity was confirmed by independent experiments performed with additional Vδ2- and Vδ1 γδ T cell lines (Figures 3, 7, 8, Supplementary Figure 2; for optimal

FIGURE 5 | Enhanced PGE2 release and COX-expression of TRAIL-R4 knockdown Colo357 cells in comparison to control cells. (A) PGE2 release was determined in the supernatant of control KD–and two different TRAIL-R (TR) 4 KD (a) or (b)—Colo357 cells after overnight culture. (B,C) In parallel, cells were intracellularly stained with anti-COX-1 and anti-COX-2 mAb and analyzed by flow cytometry. The expression is shown as fold change in median fluorescence intensity (MFI) relative to isotype control. Bars represent mean ± SD of three independent experiments. Significances are shown as P-value; * = P < 0.05 and ** = P < 0.01.
quantification and simplified presentation in Figures 3B, 7, 8, cytotoxic capacity was calculated 4 hrs after addition of γδ T cell lines as a decrease in fold change of CI compared to the control sample without effectors cells and to maximal lysis with Triton-X-100. Beside Colo357 and MDA-MB-231 cells, TRAIL-R4 was also transiently knocked-down via siRNA in HeLa cells (Supplementary Figure 1C). Importantly, all stable and transient TRAIL-R4 knockdown cells presented a significantly reduced sensitivity against γδ T cell-mediated cytotoxicity (Figure 2B). As shown in Supplementary Figure 1C, TRAIL-R1 was slightly regulated, whereas TRAIL-R2/3 were not affected by knockdown of TRAIL-R4 in HeLa cells. Of note, the results showing the potential regulatory role of tumor cell-expressed TRAIL-R4 on γδ T cell-induced cytotoxicity were similar for both γδ T cell-populations-derived cell lines. The importance of TRAIL-R4 as a regulatory protein was underlined by results demonstrating an enhanced γδ T cell-mediated cytotoxicity toward TRAIL-R4 knockin (KI) HeLa cells (Supplementary Figure 3). Since V81 γδ T cells comprise the main population in PDAC patients (34), we focused our study on these γδ T cell-subset. As TRAIL-R4-KD cells were very sensitive to TRAIL-treatment (Figures 1D,E), the diminished sensitivity of these cells to γδ T cells-mediated cytotoxicity suggested that TRAIL did not play a pivotal role in killing of tumor cells by γδ T cells. In accordance, neutralization of TRAIL via anti-TRAIL antibody or TRAIL-R2-Fc showed no effect on lysis of either control or of TRAIL-R4-KD cells (Figure 3, and data not shown). A negligible role of the TRAIL-mediated lysis of control- or TRAIL-R4-KD Colo357 cells by γδ T cells was confirmed by additional independent experiments. These results showed an insignificant effect on the Colo357 killing by γδ T cells in the presence of pan-caspase inhibitor zVAD-fmk (Figure 3B). In addition, knockdown of death-inducing TRAIL receptors, TRAIL-R1 or TRAIL-R2 in Colo357 cells, had minimal (TRAIL-R1) or no (TRAIL-R2) influence on the sensitivity of these cells to V81 T cell-mediated cytotoxicity (Supplementary Figure 4).

Unlike Colo357 and MDA-MB-231 cells, PancTu1 cells showed high sensitivity to the lysis by V81 γδ T cells in both control and TRAIL-R4-KD shRNA cells (Supplementary Figure 5). In contrast to the cytotoxicity of V81 T cells, the V82 T cell-mediated cytotoxicity against PancTu1 cells was very weak in the absence of enhancers of γδ T cell-cytotoxicity such as bsAb (48). Coculture of PancTu1 cells with V81 γδ T cells resulted in their lysis which was not affected by either neutralization of TRAIL or knockdown of TRAIL-R1 or TRAIL-R2 (Supplementary Figures 5, 6).

**Knockdown of TRAIL-R4 Enhances Expression of COX-1 and COX-2 and the Secretion of PGE2**

As cytotoxic activity of V81 T cells against TRAILR4-KD tumor cells—except for PancTu1 cells—was significantly impaired, we asked whether the down-regulation of TRAIL-R4 in these cells led to an altered expression of molecules involved in T cell-mediated cytotoxicity. The adhesion molecule CD44 was reported to be highly expressed on different tumor cells and to suppress T cell-mediated immune responses (57, 58). Our data revealed that CD44 was weakly expressed on Colo357 cells and only slightly up-regulated in TRAIL-R4-KD cells (Figure 4A). The interaction of CD54 and its corresponding ligand CD11a/CD18 expressed on γδ T cells is responsible and therefore essential for triggering cytotoxic function in γδ T cells (40, 59). However, CD54 was rather up-regulated than down-regulated in TRAIL-R4-KD cells (Figure 4A). In addition, the costimulatory receptors CD80/CD86, CD154, or MICA/MICB were very weakly expressed on Colo357 cells and did not significantly differ between control- and TRAIL-R4-KD Colo357 cells (Figure 4A). Furthermore, the expression of death receptors CD95, TNFR1, TRAIL-R1, and TRAIL-R2 as well as of TNFR2 and TRAIL-R3 did not significantly differ between control- and TRAIL-R4-KD cells (Figure 4B, Supplementary Figure 1C).

In conclusion, these data suggest that mechanisms other than changes in expression of adhesion- or co-stimulatory molecules and cell death receptors account for the enhanced resistance of TRAIL-R4-KD cells to γδ T cell-induced cytotoxicity.

One prominent defense mechanism that tumor cells developed to counteract the cytotoxic activity of T cells is the secretion of PGE2, which can diminish both the cytotoxicity and the proliferation of T cells (48, 60). Interestingly, we found that knockdown of TRAIL-R4 in Colo357 cells resulted in a significant enhancement of PGE2 secretion (Figure 5A). The PGE2 synthesis is mainly regulated by two enzymes, the constitutively active COX-1 and an inducible enzyme COX-2 (61, 62). To study whether the knockdown of TRAIL-R4 impacts on COX-1- and COX-2-expression, we compared the intracellular expression of both enzymes in control- and TRAIL-R4-KD cells by flow cytometry. We found that both, control and TRAIL-R4-KD cells expressed COX-1 as well as COX-2 (Figures 5B,C). However, TRAIL-R4-KD cells showed enhanced levels of both enzymes. Particularly COX-2 expression was strongly and significantly up-regulated in TRAIL-R4-KD cells compared to control cells (Figure 5C, Supplementary Figures 7A,B). In agreement with our previously published data, PancTu1 cells do not express COX-2 ([48] and Supplementary Figure 7A], whereas MDA-MB-231 cells express low levels of COX-2 (Supplementary Figure 7B). Of note, knockdown of TRAIL-R4 in PancTu1 cells did not change either their sensitivity to V81 T cell-mediated cytotoxicity or the COX-2 expression (Supplementary Figure 5), consistent with the postulated role of COX-2 in the inhibition of V81 T cells-induced cytotoxicity toward Colo357 TRAIL-R4-KD cells. Knockdown of TRAIL-R4 in MDA-MB-231 cells resulted in a slight increase of COX-2 expression which explains the moderate resistance of TRAIL-R4-KD cells toward γδ T cell-mediated lysis compared to TRAIL-R4-KD Colo357 cells (Figure 2B, Supplementary Figure 7B).

To study the putative mechanisms responsible for the observed increased expression of COX-2 in TRAIL-R4-KD Colo357 cells, we treated these cells as well as control cells with inhibitors of various signal transduction pathways known to play a role in TRAIL-R-induced signaling and determined their impact on COX-2 expression. Consistent with the flow cytometry data (Figure 5C), Western blot analyses of whole cell lysates showed marked upregulation...
of the cellular levels of COX-2 in TRAIL-R4-KD cells compared to the control cells (Figures 6A,B). The inhibition of AKT by MK2206 did not change the COX-2 level, whereas the inhibition of proteasome by MG-132 strongly upregulated the expression of COX-2 in both control- and TRAIL-R4-KD cells. Importantly, inhibition of MAP-kinases ERK1/ERK2 by U0126 severely reduced the amounts of COX-2 in TRAIL-R4-KD cells (Figures 6A,C). In agreement, Western blot analyses performed using phosphorylation/activity status-detecting antibodies revealed that TRAIL-R4-KD cells are characterized by strongly up-regulated activity of ERK1/ERK2 with no observed changes in the overall cellular levels of these kinases (Figures 6A–C). In contrast, HeLa TRAIL-R4-KI cell line showed a lower activity of ERK1/ERK2 (Supplementary Figure 7D).

A possible explanation for the weak lysis of TRAIL-R4-KD cells in comparison to control Colo357 cells by γδ T cell lines could be due to an enhanced PGE2 release by TRAIL-R4-KD cells as already indicated in Figure 4A. To proof this hypothesis, we cocultured Vδ1 T cell lines from different healthy donors (n = 4) and PDAC patients (n = 2) with either control- or TRAIL-R4-KD cells and compared the cytotoxic potential of Vδ1 T cell lines toward both cell lines concomitantly measuring the PGE2 release (Figures 7A,B). All applied Vδ1 T cell lines induced a partial lysis of control Colo357 cells, while TRAIL-R4-KD Colo357 cells were not lysed, as demonstrated by significantly higher CI values compared to control cells (Figure 7A). These results were independent of the Vδ1 T cell line used and the co-expression of specific Vγ-chains (Figure 7A, closed symbols: Vδ1 T cells co-expressing Vγ2, 3 or 4 chain, open symbols: Vδ1 Vγ8 T cells), indicating a lower capacity of different Vδ1 T cell-subsets to lyse TRAIL-R4-KD cells compared to control cells. Interestingly, this lower capacity was accompanied by a significantly enhanced PGE2 release by TRAIL-R4-KD cells cocultured with Vδ1 T cell lines in comparison to control Colo357 cells (Figure 7B). Our results were confirmed by additional independent experiments demonstrating that the addition of exogenous PGE2 decreased the Vδ2- as well as Vδ1 γδ T cell-mediated lysis of control Colo357 cells to a similar level as observed with TRAIL-R4-KD (Figure 7C).

![FIGURE 6](image_url) MAP Kinases ERK1/ERK2 regulate COX-2 level in Colo357 cells. Control KD- as well as TRAIL-R4 KD [TR4 KD (a)] Colo357 cells were treated with four different inhibitors [MG-132 (proteasome inhibitor), U0126 (MEK inhibitor), MK2206 (AKT1,2,3 inhibitor)] for 8h. (A) The protein levels of COX-2, phospho-ERK1/2 and ERK were detected in whole cell lysates via western blot. (B) The basal level of the different proteins in both TR4 KD- (a, b) and control Colo357 cells is shown. (C) The correlation between COX-2, phospho-ERK1/2 and ERK1/2 in control KD and TR4 KD (b) cells is presented.
Recently, we demonstrated that the Vδ2-expressing γδ T cells exert their cytotoxic activity against PDAC cells mainly via granzyme B released from cytolytic granules (34). The granzyme B release can be drastically enhanced by an addition of bsAb leading to a strong enhancement of the Vδ2 T cell-cytotoxicity (34, 53). Since cells with TRAIL-R4-KD were almost completely refractory to cytotoxic activity of Vδ1 T cells, we next investigated whether TRAIL-R4-KD cells could negatively influence the secretion of granzyme B by Vδ1 T cells. Indeed, we found that Vδ1 T cells released lower amounts of granzyme B when cocultured with TRAIL-R4-KD than with control cells (Figure 8A, med, left, and right panel). The addition of bsAb [HER2xCD3] significantly enhanced the granzyme B release and as a consequence the Vδ1 T cell-mediated lysis of control cells and TRAIL-R4-KD cells (Figures 8A,C). Since the inhibition of granzyme B release by Vδ1 T cells cocultured with TRAIL-R4-KD cells was accompanied by an enhanced PGE2 release of the latter, we additionally analyzed...
the effects of COX-1/2 inhibitor Indomethacin on granzyme B and PGE2 release. While Indomethacin significantly reduced the PGE2 release by control- as well as by TRAIL-R4-KD Colo357 cells, it had no impact on the granzyme B release by any of the cells. Conversely, while the addition of bsAb clearly enhanced the granzyme B release by Vδ1 T cells, it did not significantly alter PGE2 release by the tumor cells (Figure 8B). The latter could be explained by the observation that the treatment of Colo357 cells with bsAb did not alter the expression of neither COX-1 nor COX-2 in tumor cells (Supplementary Figure 7C). Importantly, concomitant administration of bsAb and Indomethacin enhanced the Vδ1 T cell-cytotoxicity toward control- and TRAIL-R4-KD cells more effectively than the separate administration of either substance alone (Figure 8C and data not shown). The application of bsAb together with the selective inhibitors of either COX-1 (valeryl salicylate - VS-) or COX-2 (DuP697) strongly increased the sensitivity of both control and TRAIL-R4-KD cells toward Vδ1 T cell-lysis. However, these effects were more pronounced in TRAIL-R4-KD cells than in control Colo357 cells suggesting an enhanced release of PGE2 by these cells constitute an important defense mechanism (Figure 8C). These data were confirmed using HeLa and MDA-MB-231 TRAIL-R4-KD cells which in combination with bsAb showed similar tendency (data not shown).

Taken together, we demonstrated that γδ T cell-mediated lysis of COX-2 expressing tumor cells is regulated by TRAIL-R4. Knockdown of this receptor enhanced the synthesis and

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**FIGURE 8 |** Increased Vδ1 T cell-cytotoxicity against Colo357 cells by bsAb [HER2xCD3] is further enhanced by COX inhibitors. Ten thousand control- or TRAIL-R4-KD Colo357 cells were incubated for 24 h in 96-well E-plats in medium. After 23 h, a final concentration of 1 µg/mL bsAb [HER2xCD3] or 50 µM Indomethacin alone or in combination or alternatively in combination with 30 µM DuP697 or 2.5 mM valeryl salicylate as indicated were added. After 24 h, Vδ1 T cell lines of six different donors with an E/T ratio of 25:1 and a final concentration of 12.5 U/mL IL-2 were cocultured. (A) The release of granzyme B or (B) PGE2 were measured after 24 h by ELISA and (C) Vδ1 T cell-mediated cytotoxicity against Colo357 cells by RTCA shown as decrease in Cell Index (CI) fold change calculated with the formula mentioned in Figure 7. Black bars represent mean of experiments. Significances are shown as P-value; ** = P < 0.01.
release of PGE2 by the tumor cells and reduced the release of
granzyme B by cocultured Vδ1 T cells thereby inhibiting Vδ1 T
cell-cytotoxicity. The application of bsAb which enhanced γδ T
cell-cytotoxicity and granzyme B release together with COX
inhibitors restored the sensitivity of Colo357 cells against γδ T
cell-mediated cytotoxicity.

**DISCUSSION**

In this study, we demonstrated that TRAIL-R4 expressed by
tumor cells regulates the cytotoxic activity of γδ T cells.

γδ T cells are attractive effector cells for T cell-based
immunotherapy. Although clearly both Vδ1 and Vδ2 T cells
are important for tumor immune surveillance, so far most of
the studies have been dedicated to Vδ2 T cells. The preferential
interest in Vδ2 T cells can be explained by (i) well-characterized
antigens, (ii) the ease to expand these T cells in vitro under good
manufacturing practice conditions for adoptive transfer, (iii) the
application of licensed drugs such as aminobisphosphonates and
IL-2 to activate these cells in vivo, (iv) availability of bispecific
antibodies as enhancer for Vγ9Vδ2 γδ T cell-mediated lysis of
tumor cells and (v) high plasticity of Vδ2 T cells by combining
the features of both innate and adaptive immunity ([30, 63–68]
for review).

In contrast, the functions of Vδ1 T cells are less well-
understood. Importantly, however, these cells are enriched in
the peripheral blood of PDAC patients (34) and infiltrate malignant
pancreatic and ovarian tissues (unpublished observation). Our
study revealed a superior Vδ1 T cell-cytotoxicity compared to
Vδ2 T cell-cytotoxicity from the same donors against PGE2-
secreting cancer cells. We also found that the cytotoxic activity
of these cells is less prone to inhibition by PGE2, added exogenously
to the coculture of Vδ1 T cells with tumor cells, than Vδ2 T cells.
The difference of PGE2-effects on cytotoxicity of Vδ1- and Vδ2 T
cells could be due to a weaker expression of prostaglandin E2
(PTGGER2) and E4 (PTGGER4) receptors on Vδ1 T cells than on
Vδ2 T cells (data not shown). However, our study demonstrated
that even though these receptors are weakly expressed, their
levels are sufficient to negatively influence Vδ1 T cell-cytotoxicity
against cancer cells which highly express the PG-synthetase COX.
An autocrine PGE2 effect on PDAC cells (69) can be excluded due
to the lack of prostaglandin receptor expression on these tumor
cells (data not shown). Ligation of PGE2 with E2 and E4 receptors
on γδ T cells initiates activation of the transcription factor cAMP
responsive element binding (CREB) and nuclear factor κB (NF-
κB) which results in the release of IFN-γ and TNF-α (48, 61, 70).
Both cytokines are produced by the different γδ T cell subsets
after activation and are able to enhance intracellular COX-2
expression in mesenchymal stem cells and in cancer cells as
recently described by us and others for Vδ2 T cells (48, 71). Aside
from IFN-γ and TNF-α, cytoytic mediators such as granzymes
and TRAIL are produced by γδ T cell-subsets and used for killing
a broad range of tumor cells (34, 54, 55, 72). Particularly, the
involvement of soluble TRAIL in the lysis of lung- and colon-
and breast cancer cells by Vδ2 T cells was reported recently
(54, 72–74).

Interestingly, in addition to the cell death induction, TRAIL
has been shown to upregulate COX-1 activity and PGE2 secretion
in cancer cells (75). Therefore, we were interested to know
whether the TRAIL/TRAIL-R system might play a role in the
sensitivity of tumor cells toward γδ T cell-cytotoxicity.

We found that neither the knockdown of TRAIL death
receptors in PDAC cells (e.g., PancTuI- and Colo357 cells) nor
the neutralization of TRAIL significantly impaired the killing
potential of Vδ1 T cells. This suggests a minor role of TRAIL
in the cytotoxic activity of these cells against the studied PDAC
cells. In agreement, knockdown of anti-apoptotic TRAIL-R4
in some tumor cell lines did not enhance γδ T cell-mediated
 cytotoxicity, while strongly sensitized these cells to the treatment
with recombinant TRAIL. Unexpectedly, however, it rendered
these cells even more resistant to both Vδ2- and Vδ1 T cell-
mediated cytotoxicity, independently of the co-expressed Vγ-chain,
which underlines the observation that all γδ T cell-subsets are
affected. These effects were associated with a reduced release
of granzyme B by γδ T cells and an enhanced PGE2-production
by TRAIL-R4-KD. The latter could be explained by the strong
intracellular up-regulation of COX-2 expression in TRAIL-R4-
KD cells and by an enhancement of expressed COX-1 in these
cells; both cyclooxygenases are essential enzymes in the PGE2-
synthesis. The expression of COX-2 is regulated by various signal
transduction pathways including MAP Kinases ERK1/ERK2 (76).
Importantly, we found that ERK-activity was strongly increased
in TRAIL-R4-KD cells and its inhibition reduced the level of
COX-2 in these cells to the level observed in the control.
This suggests that increased ERK-activity is responsible for the
upregulation of COX-2 expression in TRAIL-R4-KD cells. The
reason why inhibition of TRAIL-R4 results in an increased
ERK-activity is yet to be elucidated. Previously, we have shown
that stimulation of Colo357 cells with TRAIL induces ERK-
activity and this process requires the activity of apoptotic caspases
(77, 78). Such caspase-dependent TRAIL-R-mediated induction
of ERK-activity was reported also in other tumor cells (1).
As many other tumor cells, Colo357 cells express small amounts of
TRAIL (data not shown). It is therefore likely that the binding
of tumor cell-derived TRAIL in TRAIL-R4-KD cells to TRAIL-
R1/R2 results in a constitutively increased activity of caspases,
which is too weak to induce cell death, but strong enough to
activate ERK. In agreement, our unpublished data revealed that
inhibition of caspases by zVAD-fmk in Colo357 TRAIL-R4-KD
cells decreased COX-2 expression. However, the coculture of
these cells with γδ T cells, in the presence of zVAD-fmk, did not
significantly affect the tumor cell lysis. Interestingly, coculture
of Colo357 cells with Vδ1 T cells enhanced PGE2 secretion,
and this effect was more pronounced in cells with knockdown
of TRAIL-R4 than in the control cells. In contrast to the obviously
negligible role of TRAIL on Vδ1 T cell-cytotoxicity, arguably this
might point to the role of TRAIL secreted by Vδ1 T cells on the
enhancement of resistance of the studied tumor cells, especially
those cells with TRAIL-R4-KD. Thus, small amounts of TRAIL
secreted by Vδ1 T cells might slightly induce caspase-activity,
again too weak to induce cell death yet strong enough to activate
ERK leading to increased expression of COX-2 and consequently
increased PGE2 production.
Consistent with the postulated role of COX-2 in the regulation of V51 T cell-cytotoxicity toward control and TRAIL-R4-KD or TRAIL-R4-K1 cells, we found that blocking of COX-2-activity by selective COX-2 inhibitor DuP697 rendered Colo357 cells sensitive to V51 T cell-mediated cytotoxicity.

In addition to COX-2, COX-1 seems also to play a role in TRAIL-R4-KD-induced inhibition of V51 T cell-cytotoxicity, as the selective COX-1 inhibitor valeryl salicylate also increased the lysis of Colo357 cells and reduced PGE2 secretion. COX-1 has been shown to be up-regulated by TRAIL and this effect was accompanied by activation of caspases and/or NFκB and led to a significant increase in PGE2 (69, 75, 79, 80). Inhibition of PGE2 synthesis represents a therapeutic aim in treatment of many disorders, among them cancer (81–83).

Since COX-1 is present in all tissues, COX-1 inhibition would affect the whole body. Therefore, the development of PGE2-inhibitors for clinical usage is focused on COX-2. DuP697 was the paradigm for the design of further COX-2 inhibitors such as Celecoxib which are included in clinical trials combined with other therapies [https://clinicaltrials.gov: COX-2 inhibitors (81–83)]. In addition to enhanced PGE2-secretion, TRAIL-R4-KD cells inhibited granzyme B release by γδ T cells. While COX-inhibitors could suppress PGE2 release by tumor cells, they did not increase the secretion of granzyme B by cytotoxic cells. Importantly, the concomitant usage of COX2-inhibitor with bsAb completely restored the killing capacity of γδ T cells toward TRAIL-R4-KD cells.

BsAb are a class of targeted biologics which are of great interest in cancer immunotherapy to target effector cells to the tumor site (84, 85). In this context, bsAb [HER2xCD3] which targets CD3-expressing V51 T cells to HER2-expressing tumor cells seems to be very effective (34, 56). γδ T cells have raised substantial interest for immunotherapy based on their capacity to kill tumor cells, in a HLA-independent manner, which are often described to be resistant to radio- and chemotherapy (34, 53, 86, 87). A combined therapy of COX-2 inhibitors together with bsAb could be an attractive therapeutic option for COX-2 expressing tumors with downregulated expression of TRAIL-R4.

Our data suggests that TRAIL-R4 might play a decisive role in the immune surveillance. This could explain the observed downregulation of this receptor in different tumors which in some cases correlates with poor patients’ prognosis (22, 28, 29, 88, 89). For PDAC, our own unpublished data revealed that TRAIL-R4 is almost exclusively localized to intracellular compartments instead of the plasma membrane. This could support the proposed scenario that TRAIL present in the tumor microenvironment, in the absence of plasma membrane expressed anti-apoptotic TRAIL-R4, would enhance TRAIL-R1/-R2-mediated COX2 expression thereby inhibiting γδ T cell activity.

Further studies are necessary to prove the generality of our findings. However, the previously unrecognized function of TRAIL-R4 in shaping the immune response strongly demands future research on the regulatory role of this receptor particularly in context of the other TRAIL receptors.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

PBMC were isolated from leukocyte concentrates obtained from healthy blood donors and provided by the Department of Transfusion Medicine of the University Hospital Schleswig-Holstein (UKSH) in Kiel, Germany. In addition, heparinized blood from PDAC patients was obtained from the Department of General Surgery of the municipal hospital. In accordance with the Declaration of Helsinki, written informed consent was obtained from all donors, and the research was approved by the relevant institutional review boards (ethic committee of the Medical Faculty of the CAU Kiel, code number: D445/18).

AUTHOR CONTRIBUTIONS

DT, CG, H-HO, J-PG, and DW performed experiments. DT, CG, H-HO, and J-PG helped to design the study. MP designed and provided the bspecific antibodies. DT, CG, and H-HO wrote parts of the manuscript. TB provided blood from pancreatic cancer patients and contributed to the discussion. DK and MP contributed to the discussion and the composition of the manuscript. AT and DW designed the project and wrote and finalized the manuscript.

FUNDING

This work was supported by the Medical Faculty (F357915, DW/AT) and DFG FOR2799 (WE 3559/6-1), German Cancer Aid (Project 70112281 given to AT and Mildred-Scheel-Professorship program to MP), and German Egyptian Long-term Scholarship (GERLS) given to DT.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Gökhan Alp and Sandra Ussat. We thank Liane Carstensen, Bianca Zinke, Christian Röder, and Susanne Sebens for organizing and providing blood from PDAC patients. We thank Olivier Micheau (INSERM, Dijon, France) for providing the TRAIL-R4-K1 and control HeLa cells. We also thank Dieter Adam for fruitful discussions. This work forms part of CG’s Master thesis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02044/full#supplementary-material
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.