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Regulation of PD-L1 expression by TRAIL in triple-negative breast cancer cells

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Abstract

Triple-negative breast cancer (TNBC) is an aggressive form of breast cancer that lacks targeted therapies. Previous studies have shown that TNBC cells are highly sensitive to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), making it a promising agent for treating TNBC. However, the development of TRAIL resistance limits its further clinical development, and the underlying mechanisms are not fully understood. In this study, we report the role of PD-L1 in TRAIL resistance. Specifically, we found that TRAIL treatment increases PD-L1 expression in TRAIL-sensitive cells and that basal PD-L1 expression is increased in acquired TRAIL-resistant cells. Mechanistically, we found that increased PD-L1 expression was accompanied by increased ERK activation. Using both genetic and pharmacological approaches, we showed that knockdown of ERK by siRNA or inhibition of ERK activation by the MEK inhibitor U0126 decreased PD-L1 expression and increased TRAIL-induced cell death. Furthermore, we found that knockout or knockdown of PD-L1 enhances TRAIL-induced apoptosis, suggesting that PD-L1-mediated TRAIL resistance is independent of its ability to evade immune suppression. Therefore, this study identifies a non-canonical mechanism by which PD-L1 promotes TRAIL resistance, which can be potentially exploited for immune checkpoint therapy.

Keywords

TRAIL; resistance; TNBC; ERK; PD-L1

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Author contributions

GSW conceived, MS designed, analyzed the results, and coordinated the project. JP and JYZ conducted the experiments and analyzed the data. JP and GSW wrote the original draft. JP, JYZ, and GSW reviewed and edited. All authors contributed to the article and approved the submitted version.

Conflict of interests

The authors declare that they have no conflict of interest.

Introduction

Breast cancer is the most common cancer and the second-leading cause of cancer deaths among women in the United States[1]. An estimated 281,550 women will be diagnosed with breast cancer by 2022, with 43,600 dying from the disease[1]. There are at least five distinct types of breast cancer. Among these five subtypes, triple-negative breast cancer (TNBC) accounts for 15–20% of all breast cancer cases and has the worst prognosis of any breast cancer subtype[2,3]. Patients with TNBC do not respond to conventional targeted/endocrine therapy due to a lack of estrogen/progesterone receptors and HER2 amplification[4,5]. Therefore, developing more effective treatment regimens for TNBC patients is becoming increasingly important. Despite the lack of targeted therapies for the majority of TNBC patients, TNBC cells are highly sensitive to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). This feature warrants developing TRAIL as a potential agent for TNBC therapy.

TRAIL is a TNF superfamily cytokine that selectively induces apoptosis in tumor cells without harming normal cells[6–9]. TRAIL initiates apoptosis by binding to its death receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5) to promote receptor trimerization[10–16]. This, in turn, recruits Fas-associated protein with death domain (FADD) and pro-caspase 8 to form the death-inducing signaling complex (DISC), resulting in caspase 8 activation[12,13]. The latter activates caspases 3, 6, and 7 to induce cell death. In some cells, activated caspase 8 cleaves the Bcl-2 family member Bid to generate truncated Bid (tBID) that can translocate to the mitochondria[17,18] where it initiates the mitochondrial apoptosis pathway to amplify TRAIL-induced cell death.

TRAIL's safety in cancer patients has been well established in phase I/II clinical trials[19]. However, the development of TRAIL resistance limits its further development for clinical use. Previous studies suggest that TRAIL resistance mechanisms can manifest themselves anywhere along the TRAIL signaling pathway[20]. For example, mutations in the death receptors DR4/DR5, as well as increased decoy receptor expression, have been found on the cell surface of TRAIL-resistant tumors[20]. The activation of oncogenic signaling pathways such as AKT, ERK1/2, and NF-κB can also lead to TRAIL resistance[21–23]. Furthermore, overexpression of the inhibitory protein c-FLIP at the DISC can inhibit caspase 8 activation, resulting in decreased apoptosis. In addition, upregulation of the anti-apoptotic Bcl-2 family members (e.g., Bcl-2 and Mcl-1) and Inhibitor of Apoptosis (IAP) family proteins (e.g., XIAP and survivin) can confer resistance to the TRAIL apoptosis pathway[7,24–26]. Nonetheless, further research is needed to better understand the mechanisms of TRAIL resistance in order to use it in clinical settings.

The programmed death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1) system plays a critical role in suppressing T cells in the tumor microenvironment[27]. PD-1 is an inhibitory receptor that is expressed on the surface of antigen-activated T cells, while PD-L1 is a ligand for PD-1 that is expressed on many immune cells, including T and B cells. The binding of PD-L1 or PD-L2 (another ligand) to PD-1 emits a negative signal that suppresses T cell activity[27,28]. Consequently, many tumor cells express PD-L1 and use PD-L1 to inhibit T cell function from evading immune surveillance[29]. Blocking the PD-1-PD-L1

interaction activates cytotoxic T lymphocytes, resulting in antitumor activity. Therefore, understanding how PD-L1 expression is regulated could lead to the development of better strategies to boost the immune system to improve PD-L1-based cancer therapies. A number of transcription factors, including STAT1/STAT3, HIF1/HIF2, c-Jun, Myc, and NF-κB, can regulate PD-L1 expression[30]. These transcription factors are activated by oncogenic signaling pathways such as JAK/STAT, RAS/RAF/ERK, and PI3K/ATK/mTOR, which are commonly upregulated in major cancers. Because TRAIL activates several of these oncogenic signaling pathways[31–34], understanding their relevance to PD-L1 expression will serve as the foundation for developing a cancer therapy strategy that combines both TRAIL ligand and PD-L1 antibody.

In this study, we investigated the role of PD-L1 in the TRAIL signaling pathway in TNBC cells. We found that TRAIL induces PD-L1 expression in TRAIL-sensitive cells and that basal levels of PD-L1 are elevated in TRAIL-resistant cells with increased ERK activation. Importantly, we found that either directly knocking down or knocking out PD-L1 expression with siRNA or CRISPR/Cas9, or indirectly inhibiting the ERK pathway with siRNA and the MEK inhibitor U0126, sensitizes TNBC cells to TRAIL-induced apoptosis. Therefore, our results suggest a non-canonical mechanism by which PD-L1 confers TRAIL resistance in TNBC cells.

Materials and Methods

Cell lines and cell culture

The TNBC cell lines MDA-MB-231 (MDA231) and SUM159 were acquired from the American Type Culture Collection (ATCC) (Rockville, MD, USA). MDA231 PD-L1 knockout cells were kindly gifted from Dr. Haidong Dong (Mayo Clinic) as described previously[35,36]. MDA231 and SUM159 cells were cultured in Dulbecco's modified Eagle's medium (DMEM). All cells were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) and maintained at 37°C in a humidified 5% CO_2 atmosphere.

Reagents and antibodies

PVDF membranes (catalog no. IPVH00010) and actin antibody (catalog no. A1978) were purchased from Sigma-Aldrich. Recombinant Human sTRAIL/Apo2L (catalog no. 310-04) was purchased from PeproTech. The MEK inhibitor U0126 (catalog no. V1121) was purchased from Promega. Trypsin-EDTA (catalog no. 25300-054), DMEM (catalog no. 11995-065), bovine serum albumin (catalog no. BP1605-100), FBS (catalog no. 10437028), goat anti-mouse alexa fluor 680 IgG (catalog no. A21058), Lipofectamine™ RNAiMAX transfection reagent (catalog no. 13778150), Opti-MEM, reduced serum medium, no phenol red, (catalog no. 11058021), P/S (catalog no. 15140122), Power SYBR Green PCR mix (catalog no. 4367659), SuperScript™ III First-Strand Synthesis System (catalog no. 18080051), goat anti-rabbit alexa fluor 680 IgG (catalog no. A21109), SuperSignal™ West PICO PLUS chemiluminescent substrate (catalog no. 34580), and TRIzol™ reagent (catalog no. 15596026) were purchased from ThermoFisher Scientific. Anti-rabbit IgG HRP-linked (catalog no.7074), anti-mouse IgG HRP-linked (catalog no. 7076), PD-L1 (405.9A11) mAB (catalog. No 29122S), cleaved caspase-8 mAB (D391) (18CB) (catalog

no. 9496S), cleaved-caspase 3 (D17S) (5A1E) mAB (catalog no. 9664S), P-ERK 1/2, P-p44/42 MAPK (T202/Y204) (197G2) mAB (catalog no. 4377S), ERK 1/2, p44/p42 MAPK mAB (catalog no. 9102S), and PARP (catalog no. 9542) were purchased from Cell Signaling. MTT was purchased from Cayman Chemical Company (catalog. No 21795). siGenome SMARTpool Human MAPK1 (catalog no. M-003555-04), siGenome SMARTpool Human MAPK3 (catalog no. M-003592-03), siGenome SMARTpool human CD274 (catalog no. M-015836-01-0010), siGenome control pool, and non-targeting (catalog no. D-001206-13-05) were purchased from Dharmacon. The protein assay dye (catalog no. 500-0006) was purchased from Bio-Rad.

Establishment of TRAIL-resistant cell lines

TRAIL-resistant MDA231 (MDA231-R) and SUM159 (SUM159-R) cells were established by gradually exposing parental MDA231 and SUM159 cells to increasing concentrations of TRAIL (5 ng/ml to 120 ng/ml) over 6 months as described previously[37,38]. Resistant cells were maintained in TRAIL (120 ng/ml). The Karmanos Cancer Institute Biobanking Core's genotyping service authenticated both parental and resistant MDA231 and SUM159 cells.

MTT assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was performed as described previously[39,40]. In brief, a total of 2000 – 10,000 cells were seeded in 96-well plates. Following overnight attachment, cells were incubated with different concentrations of the drug in 200 μl media for 24 hours, 48 hours, or 72 hours. Each well received 20 μl of MTT solution (5 mg/ml) and was incubated at 37°C for 2 hours. MTT-containing media was removed, and formazan crystals were dissolved in 100 μl DMSO. A SynergyTM-2 microplate reader (BioTek Instruments Inc., Winooski, VT, USA) was used to measure the optical density at 570 nm. The IC50 values were calculated with Microsoft Excel. Experiments were performed in triplicates.

Colony formation assay

A total of 500 cells were seeded in 24-well plates. Following overnight attachment, cells were incubated with different drug concentrations in 2 ml for 48 hours or 72 hours. Cells were then maintained in a drug-free medium for an additional 14 days. The media was changed every two days. The resulting cells were washed with PBS and fixed for 10 min in cold 100% methanol. Cells were stained with 0.25% crystal violet for 30 min and washed in water. After air drying, colonies with more than 50 cells were counted using Oxford Optronix GELCOUNT™.

Western blot analysis

Western blot analysis was performed, as described previously[41]. Briefly, cells were lysed with 1x NP40 lysis buffer containing protease and phosphatase inhibitors. Total protein was collected following a 12 min centrifugation at 12,000 rpm at 4°C. A Bio-Rad protein assay was used to determine protein concentration. A total of 20–140 μg of protein was electrophoresed in a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). The proteins were transferred to 0.45 μM polyvinylidene difluoride (PVDF) membranes and

blocked with 2% casein. The membranes were incubated at 4°C overnight with specific primary antibodies before being incubated for 1 hour with Alexa Fluor conjugated secondary antibodies. Signals were detected using an Odyssey infrared imaging system (LI-COR) at 700 nm (Li-Cor Biosciences, Lincoln, NE, USA) or Enhanced Chemiluminescence (ThermoFisher Scientific, catalog no. 34580).

RNA isolation, cDNA synthesis, and Real-Time PCR

Total RNA was isolated using TRIzol (Life Technologies), and cDNA was synthesized using the Invitrogen SuperScript III Reverse Transcriptase kit (ThermoFisher Scientific) with 1μg - 5μg total RNA and random primers. The following primer sequences were used for semi-quantitative and Real-Time PCR: GAPDH forward (5'-ATC AAG AAG GTG GTG AAG CAG-3', GAPDH reverse (5'-TGT CGC TGT TGA AGT CAG AGG-3', PD-L1 forward (5'-GCT GCA CTA ATT GTC TAT TGG GA-3', PD-L1 reverse (5'-AAT TCG CTT GTA GTC GGC ACC −3'. PD-L1 primer sequences were obtained from Invitrogen. The Power SYBR Green PCR mix (ThermoFisher Scientific) was used for real-time RT-PCR on the Step One Plus Real-Time PCR System. The thermal cycling conditions were as follows: 95°C for 10 min, followed by forty cycles of 95°C for 15 sec and 60°C for 1 min. The ΔΔCt method was used to determine relative RNA levels, with GAPDH as the internal control. Each sample was tested in duplicate, and the results shown are representative of at least three independent experiments.

siRNA transfection for ERK or PD-L1 knockdown

siRNA knockdown was performed, as described previously[42]. Specifically, cells were seeded in 60 mm plates overnight. The following day, cells were transfected with ERK (ERK1 and ERK2), PD-L1, or control siRNA (Dharmacon) using LipofectamineTM RNAiMAX transfection reagent (ThermoFisher Scientific) as directed by the manufacturer. After 48 hours, the cells were treated or left untreated at the indicated time points. After treatment, cells were harvested to confirm ERK1/2 or PD-L1 expression by western blot analysis. Cell viability was then determined using MTT or colony formation assays in both treated and untreated cells.

Statistical analysis

Microsoft Excel was used to analyze all the data. LI-COR or ImageJ was used to perform densitometry analyses. The data were presented as mean ± standard deviation (SD). The Student's t-test was used to compare groups.

Results

PD-L1 expression is increased by TRAIL in TRAIL-sensitive cells, while basal PD-L1 levels are elevated in TRAIL-resistant TNBC cells.

Because TRAIL plays a role in immune surveillance, we asked if TRAIL treatment affects PD-L1 expression in TNBC cells. To this end, we treated two TRAIL-sensitive TNBC cells, MDA231 and SUM159, with different doses of TRAIL for 24 hours, and the induction of PD-L1 was assessed by western blot analysis. We chose TNBC cells because TNBC cells are sensitive to TRAIL-induced apoptosis. Fig. 1A shows that PD-L1 expression is

induced in both TRAIL-treated MDA231 and SUM159 cells than in their corresponding untreated control cells. In MDA231 cells, PD-L1 was induced by TRAIL at 1.56 ng/ml, which was elevated higher with higher doses of TRAIL treatment. In SUM159 cells, PD-L1 induction by TRAIL is in a dose-dependent manner. Increased PD-L1 expression by TRAIL treatment suggests a role of PD-L1 in the TRAIL signaling pathway, possibly involving TRAIL resistance in TNBC cells.

To test if an increase in PD-L1 expression by TRAIL plays a role in TRAIL resistance, we assessed basal PD-L1 levels in two pairs of TRAIL-sensitive and TRAIL-resistant MDA231-P/MDA231-R and SUM159-P/SUM159-R cells by western blot analysis. Fig. 1B shows that MDA231-R and SUM159-R cells had higher basal PD-L1 levels than their TRAIL-sensitive counterparts, suggesting that an increase in PD-L1 expression in TRAIL-resistant MDA231 and SUM159 cells may associate with TRAIL resistance in TNBC cells.

TRAIL-induced PD-L1 expression is transcriptionally regulated

Next, we asked if increased PD-L1 expression by TRAIL occurs at the transcriptional level. We treated MDA231 and SUM159 cells with various doses of TRAIL for 24 hours, and RNAs were isolated, followed by cDNA synthesis. Real-time PCR (RT-PCR) was performed using PD-L1 primers. Fig. 2A shows that PD-L1 mRNA expression was increased in treated cells compared to untreated cells. Furthermore, we found that TRAIL-resistant MDA231-R and SUM159-R cells expressed higher PD-L1 mRNA expression than their corresponding TRAIL-sensitive control cells (Fig. 2B). These results are consistent with protein levels (Fig. 1A), indicating that PD-L1 induction by TRAIL in TRAIL sensitive cells and increased basal PD-L1 expression in TRAIL-resistant cells are transcriptionally regulated.

Increased PD-L1 expression is accompanied by increased ERK activation

Accumulating evidence suggests that TRAIL can activate several survival pathways, including ERK, to counteract apoptosis induction[21]. It is well-established that ERK pathway activation increases PD-L1 expression in major cancers[43]. Accordingly, we investigated the role of ERK activation in the regulation of PD-L1 expression. Fig. 3A shows that TRAIL treatment increased phosphorylated (activated) ERK and PD-L1 levels in MDA231 and SUM159 cells compared to untreated cells. Consistently, higher basal PD-L1 levels were accompanied by increased ERK activation in MDA231-R and SUM159-R cells than in their corresponding sensitive cells (Fig. 3B). Thus, increased PD-L1 expression accompanied by increased ERK activation suggests that TRAIL-mediated PD-L1 expression may be due to ERK-dependent mechanism.

Modulation of ERK activation decreases PD-L1 expression and increases TRAIL sensitivity

Having established that PD-L1 expression is accompanied by increased ERK activation (Fig. 3), we asked if ERK activation is required for PD-L1 expression and, subsequently, TRAIL resistance. We first tested if the inhibition of ERK activation impairs TRAIL-induced PD-L1 expression. We treated MDA231 cells with TRAIL in the presence and absence of the MEK inhibitor U0126. Fig. 4A shows that U0126 effectively inhibits TRAIL-induced ERK activation and TRAIL-induced PD-L1 expression compared to cells without U0126 treatment, suggesting that TRAIL-induced PD-L1 expression depends on activated ERK.

MTT assay revealed that U0126 enhanced TRAIL-induced loss of cell viability compared to cells treated with TRAIL alone (Fig. 4C). Moreover, U0126 treatment decreased basal PD-L1 levels (Fig. 4B) in TRAIL-resistant MDA231-R cells. Importantly, MTT and colony formation assays revealed that U0126 treatment increased TRAIL sensitivity in TRAIL-resistant MDA231-R cells (Fig. 4C and D).

Although the MEK inhibitor U0126 decreased PD-L1 expression and increased TRAIL sensitivity in MDA231 cells (Fig. 4), these results may not fully reflect the role of ERK activation in TRAIL resistance because U0126 may inhibit other kinases. To validate the role of ERK activation in PD-L1 expression and TRAIL resistance, we used siRNA to knock down ERK expression in MDA231 cells. Specifically, we transfected MDA231 cells with ERK1/2 siRNA or control siRNA, then treated the cells with TRAIL for 48 hours. We showed that ERK knockdown by siRNA against ERK1/2 decreased basal PD-L1 levels in MDA231 cells (Fig. 5A) and sensitized MDA231-R cells to TRAIL (Figs. 5B and C). Thus, these results validate the role of ERK activation in PD-L1 expression and TRAIL resistance in MDA231 cells.

Downregulation of PD-L1 has a significant impact on TRAIL sensitivity

To assess the role of PD-L1 in TRAIL-induced apoptosis, we asked if PD-L1 expression is required for TRAIL resistance. First, we employed siRNA to knock down PD-L1 expression to assess the effect of PD-L1 knockdown on TRAIL sensitivity. Fig. 6A shows that siRNA effectively knocked down PD-L1 in MDA231 cells and that PD-L1 knockdown sensitized MDA231-R cells to TRAIL compared to cells transfected with control siRNA (Fig. 6B). Then, we compared the effect of TRAIL-induced apoptosis between PD-L1 knockout MDA 231 cells and cells with intact PD-L1. We confirmed that PD-L1 knockout MDA231 cells did not express PD-L1 by western blot analysis (Figs. 7A). Importantly, we showed that PD-L1 knockout MDA231 cells were more sensitive than control cells to TRAIL-induced apoptosis (Figs. 7B and 7D). We then investigated whether PD-L1 knockout MDA231 cells responded to TRAIL at a higher apoptotic rate than control cells. Fig. 7C shows that PD-L1 knockout MDA231 cells had increased cleaved caspase 3/8 and PARP levels than control cells under the same treatment conditions. Collectively, these data strongly suggest that PD-L1 protein expression has a negative impact on the TRAIL sensitivity of TNBC cells.

Discussion

The TRAIL pathway remains an attractive target for cancer therapy due to its ability to induce cancer cell death selectively, but the development of TRAIL resistance is a significant issue that needs to be addressed. In this study, we showed that TRAIL resistance in TNBC cells is mediated in part by PD-L1, which is independent of its ability to regulate the immune checkpoint.

The mechanisms of TRAIL resistance have been studied extensively but are not fully understood. Previous studies have identified many pathways involved in TRAIL resistance, focusing on intrinsic and extrinsic apoptosis pathways[8,9]. While previous studies suggested the role of TRAIL in tumor immune surveillance[44–46], we wanted to know if TRAIL treatment affects the expression of immune checkpoint PD-L1 on tumor cells. By treating

TNBC cells with TRAIL, we found that PD-L1 is induced by TRAIL and, moreover, is increased in TRAIL-resistant cells compared to their corresponding TRAIL-sensitive counterparts. Consistently, a very recent study indicated that TRAIL could induce PD-L1 expression to promote epithelial-to-mesenchymal transition (EMT) in esophageal squamous cell carcinoma[47]. In addition to the role of TRAIL in inducing PD-L1 expression, DR5 agonist antibodies were also shown to induce PD-L1 expression to promote an immune-suppressive tumor microenvironment[48]. Although these two studies showed the induction of PD-L1 by TRAIL or the TRAIL pathway via the DR5 agonist antibodies, our study differs from these studies. While these two studies suggest a role of TRAIL-induced PD-L1 in promoting the EMT[47] or a role of DR5 antibody-induced PD-L1 in inhibiting T cell functions from influencing the tumor microenvironment[48], our study suggests that increased PD-L1 expression is part of TRAIL resistance mechanism in TRAIL-resistant cells and that PD-L1-mediated TRAIL resistance is independent of its ability to evade the immune checkpoint.

The underlying mechanism through which PD-L1 expression confers TRAIL resistance remains to be defined. Several lines of evidence suggest that PD-L1 expression can be regulated by several oncogenic signaling pathways, including ERK, AKT, and NF-κB, all commonly upregulated in major cancers[30–34]. In a previous study, we showed that the preceding oncogenic signaling pathways were concurrently correlated with TRAIL resistance in TNBC cells[21]. In this study, we showed that increased PD-L1 expression in TNBC cells was transcriptionally regulated and that higher PD-L1 levels were accompanied by increased ERK activation. Additionally, we showed that inhibiting the ERK pathway decreased PD-L1 expression while increasing TRAIL sensitivity in TNBC cells. Thus, we speculate that the activation of ERK by TRAIL increases c-Jun expression and AP-1 activity. The latter can transcriptionally induce PD-L1 expression. When PD-L1 expression increases, it can prevent TRAIL-induced apoptosis by either inhibiting caspase 8 activation or interfering with the TRAIL-DR4/5 interaction (Fig. 7E).

While the role of PD-L1 in regulating T cell function is well documented, accumulating evidence is emerging that PD-L1 plays a role in chemoresistance. It has been shown that PD-L1 inhibition can enhance chemosensitivity by promoting apoptosis in breast cancer cells[35,49]. Because TRAIL is a potent apoptosis inducer, enhancing chemosensitivity by inhibiting PD-L1 led us to hypothesize that increasing PD-L1 may play a role in TRAIL resistance in TNBC cells. As shown in this study, knockdown or knockout of PD-L1 expression significantly impacted TRAIL sensitivity in TNBC cells. We also showed that TNBC cells with PD-L1 knockdown or knockout had a higher apoptotic response rate, as evidenced by increased caspase 3/8 activation and PARP cleavage levels. Consistent with this observation, it has been shown the combination of anti-PD-L1 antibody with TRAIL effectively induced cancer cell death[50]. Thus, these results validate the role of PD-L1 expression in TRAIL resistance in TNBC cells.

In summary, levels of PD-L1 expression are correlated with TRAIL resistance in TNBC cells. ERK, which is activated by TRAIL, transcriptionally regulates PD-L1 expression. More importantly, indirect inhibition of PD-L1 expression through the ERK pathway or direct downregulation of PD-L1 expression increases TRAIL sensitivity in TNBC cells.

Based on these findings, additional research is required to determine the efficacy of combining PD-L1 inhibition and TRAIL-based therapy in TRAIL-resistant TNBC cells *in vivo*. Furthermore, functional studies focusing on the signaling pathway by which TRAIL activates ERK and subsequently increases PD-L1 expression are needed to better understand the role of PD-L1 in TRAIL resistance. We believe that these findings may be applicable to other TRAIL-resistant tumors, which warrants further investigation. Therefore, our findings suggest that targeting PD-L1 may effectively overcome TRAIL resistance in TNBC cells.

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Data Availability Statement

All representative data are contained within the article.

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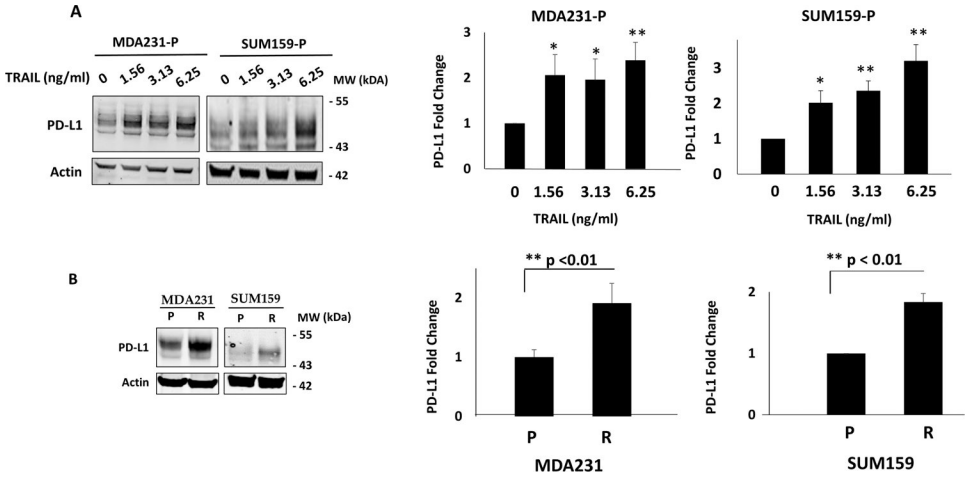

Figure 1. PD-L1 expression is increased by TRAIL and in TRAIL-resistant TNBC cells.

Western blots of PD-L1 expression. Left: Western blot images. Right: Densitometry bar graphs depicting PD-L1 fold change. (A) MDA231-P and SUM159-P cells were treated for 24 hours with the indicated TRAIL doses. (B) MDA231 and SUM159 cells. Actin was used as a loading control. 0.1% DMSO was used as solvent control. P; parental, R; TRAIL-resistant. Data are mean ± S.D. *P < 0.05; **P < 0.01; *** P < 0.001 by Student's *t-test*. Error bars represent standard deviation.

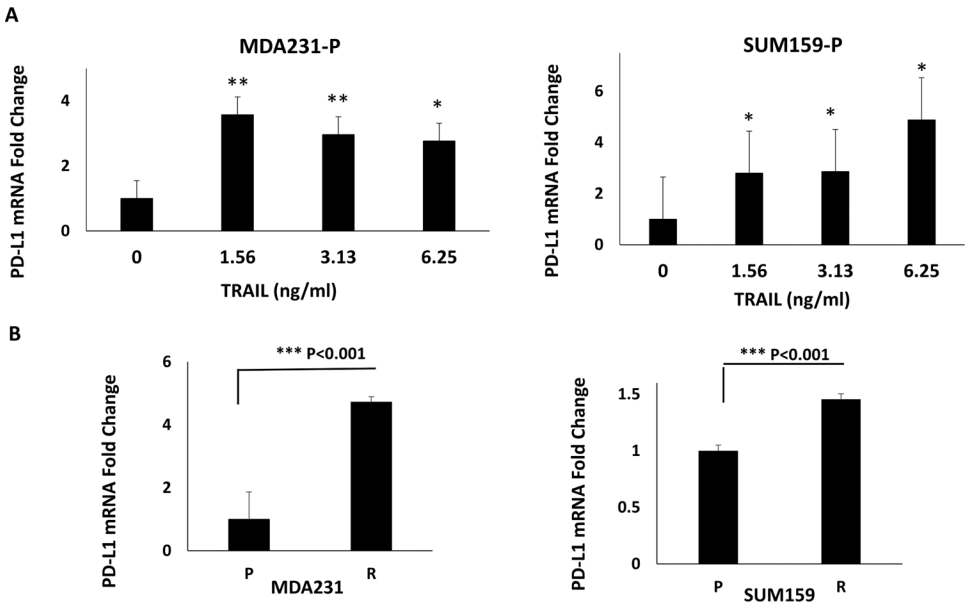

Figure 2. TRAIL-induced PD-L1 expression is transcriptionally regulated.

Real-time PCR of PD-L1 mRNA expression. (A) MDA231-P and SUM159-P cells were treated with TRAIL (1.56 ng/ml) for 24 hours. (B) MDA231 and SUM159 cells. All experiments are representative of three independent experiments. PD-L1 mRNA expression levels were normalized to GAPDH. 0.1% DMSO was used as solvent control. P; parental, R; TRAIL-resistant. Data are mean ± S.D. *P < 0.05; **P < 0.01; *** P < 0.001 by Student's *t-test*. Error bars represent standard deviation.

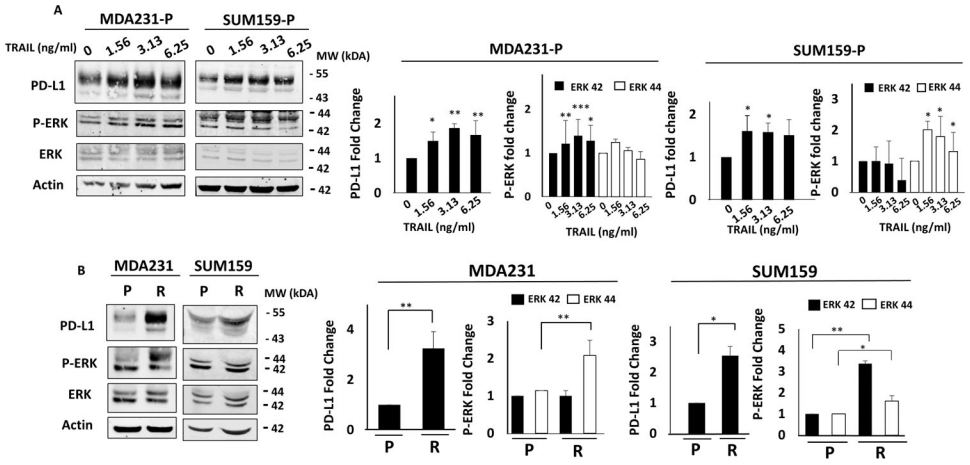

Figure 3. Increased PD-L1 expression is accompanied by increased ERK activation.

Western blots of PD-L1, ERK, and phosphorylated (activated) ERK. Left: Western blot images. Right: Densitometry bar graphs depicting protein fold change. (A) MDA231-P and SUM159-P cells were treated for 24 hours with the indicated TRAIL doses. (B) MDA231 and SUM159 cells. Actin was used as a loading control. 0.1% DMSO was used as solvent control. P; parental, R; TRAIL-resistant. Data are mean ± S.D. *P < 0.05; **P < 0.01; *** P < 0.001 by Student's *t-test*. Error bars represent standard deviation.

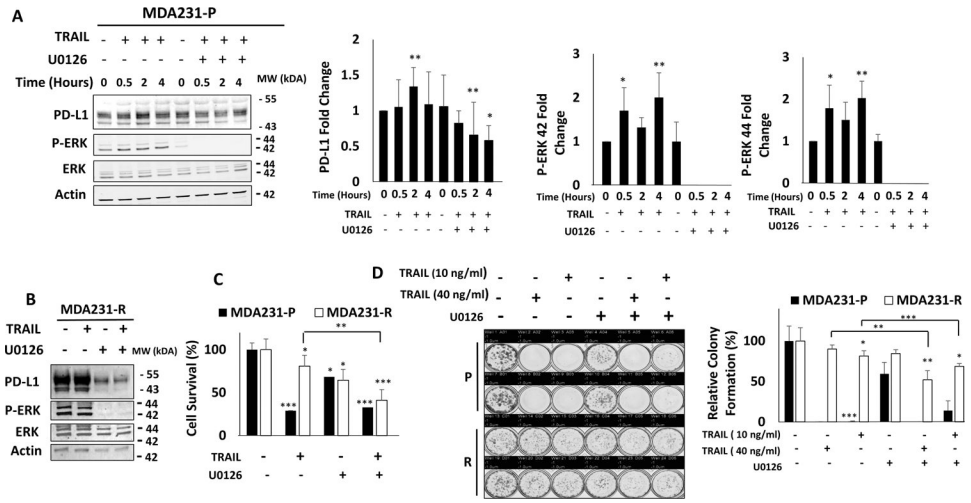

Figure 4. MEK inhibitor U0126 inhibits ERK activation, reduces PD-L1 expression, and increases TRAIL sensitivity.

(A) MDA231-P cells were treated with U0126 (10 μM), TRAIL (1.56 ng/ml), or the combination at the indicated time points. Left: Western blot images of PD-L1, ERK, and phosphorylated (activated) ERK levels. Right: Densitometry bar graphs depicting protein fold change. (B) MDA231-R cells were treated with TRAIL (50 ng/ml), U0126 (10 μM), or the combination for 48 hours. Western blot images of PD-L1, ERK, and phosphorylated (activated) ERK levels. (C) The MTT assay was performed to determine the survival of MDA231 cells following the treatment described in Fig. 4B. (D) Left: Colony formation assay MDA231 cells treated with TRAIL (10 ng/ml or 40 ng/ml), U0126 (10 μM), or the combination for 72 hours. Right: Densitometry bar graph depicting relative colony formation. Actin was used as a loading control. 0.1% of DMSO was used as solvent control. P; parental, R; TRAIL-resistant. Data are mean ± S.D. *P < 0.05; **P < 0.01; *** P < 0.001 by Student's *t-test*. Error bars represent standard deviation.

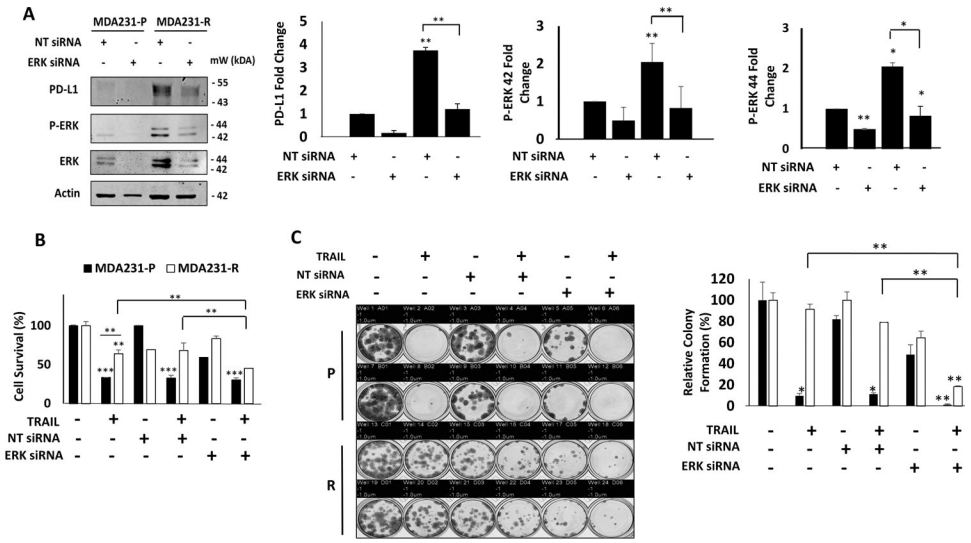

Figure 5. Knockdown of ERK decreases PD-L1 expression and increases TRAIL sensitivity.

MDA231 cells were transfected with ERK or non-target siRNAs for 48 hours. (A) Western blot of ERK, phosphorylated ERK, and PD-L1 levels in MDA231 cells. Left: Western blot images. Right: Densitometry bar graphs depicting protein fold change. (B) MTT assay was performed to determine cell viability in MDA231 cells with ERK knockdown as in (A). The resulting cells were treated with TRAIL (50 ng/ml) for 48 hours. (C) Colony formation assay and quantification of MDA231 cells that had been transfected with ERK1/2 siRNA or nontarget siRNA and treated for 72 hours with TRAIL (25 ng/ml for P; 50 ng/ml for R). Left: Colony formation assay. Right: Densitometry bar graph depicting relative colony formation. Actin was used as the loading control. 0.1% DMSO was used as solvent control. NT; non-target, P; parental, R; TRAIL-resistant. Data are mean ± S.D. *P < 0.05; **P < 0.01; *** P < 0.001 by Student's *t-test*. Error bars represent standard deviation.

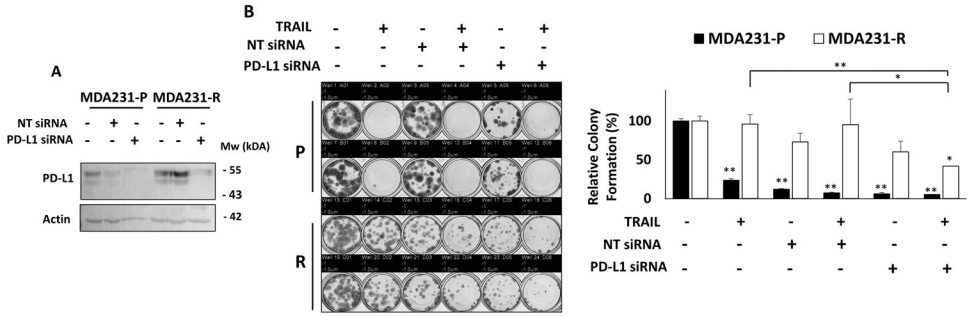

Figure 6. Knockdown of PD-L1 increases TRAIL sensitivity.

MDA231 cells were transfected with PD-L1 or non-target siRNAs for 48 hours. (A) Western blot of PD-L1 in MDA231 cells. (B). Colony formation assay and quantification of MDA231 cells that had been transfected with PD-L1 siRNA or nontarget siRNA and treated for 72 hours with TRAIL (25 ng/ml for P; 50 ng/ml for R). Left: Colony formation assay. Right: Densitometry bar graph depicting relative colony formation. Actin was used as the loading control. 0.1% DMSO was used as solvent control. NT; non-target, P; parental, R; TRAIL-resistant. Data are mean ± S.D. *P < 0.05; **P < 0.01 by Student's *t-test*. Error bars represent standard deviation.

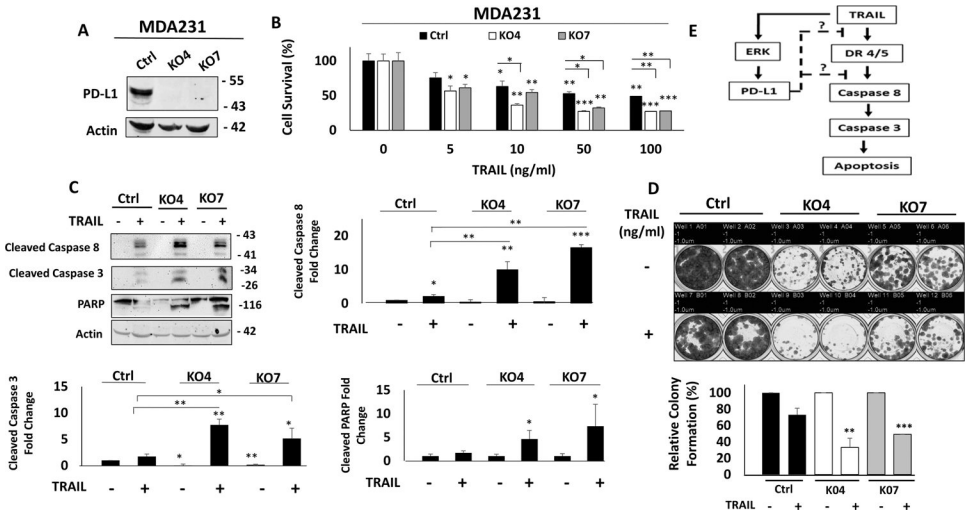

Figure 7. Loss of PD-L1 has a significant impact on TRAIL sensitivity.

PD-L1 knockout was achieved by CRISPR-Cas9 technology, as previously described[35,36]. (A) PD-L1 knockout was confirmed by Western blot analysis. (B) MTT assay was used to determine the cell viability of MDA231 cells with PD-L1 knockout after 72 hours of TRAIL treatment at the indicated doses. (C) Western blot analysis of cleaved caspase 3, 8, and PARP levels in MDA231 cells after a 10-hour TRAIL treatment (10 ng/ml). Top left: Western blot image. Top right, bottom left, and bottom right: Densitometry bar graphs depicting protein fold change. (D) Colony formation of MDA231-control/KO4/KO7 cells treated with TRAIL at 2.5 ng/ml for 72 hours. Top: Colony formation assay. Bottom: Densitometry bar graph depicting relative colony formation. Actin was used as a loading control. 0.1% DMSO was used as solvent control. Ctrl; control, KO4; PD-L1 knockout clone 4, KO7; PD-L1 knockout clone 7. Data are mean ± S.D. *P < 0.05; **P < 0.01; *** P < 0.001 by Student's *t-test*. Error bars represent standard deviation. (E) A model for the role of PD-L1 in TRAIL resistance.