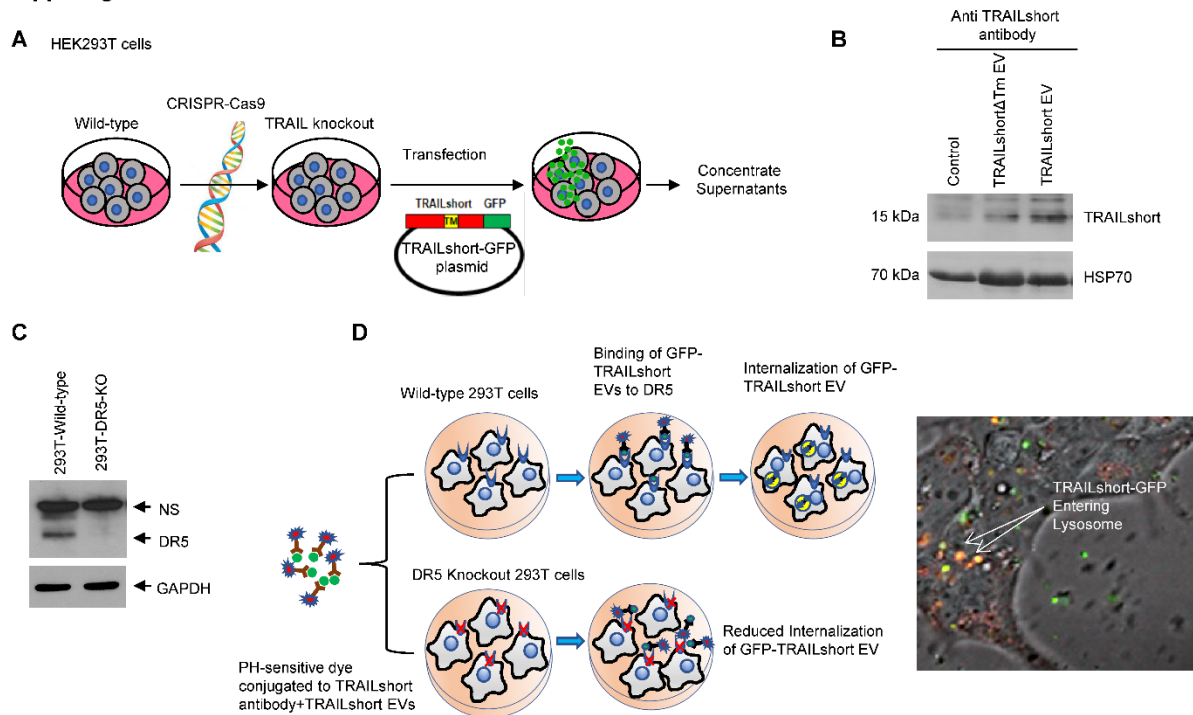


Supplemental Materials

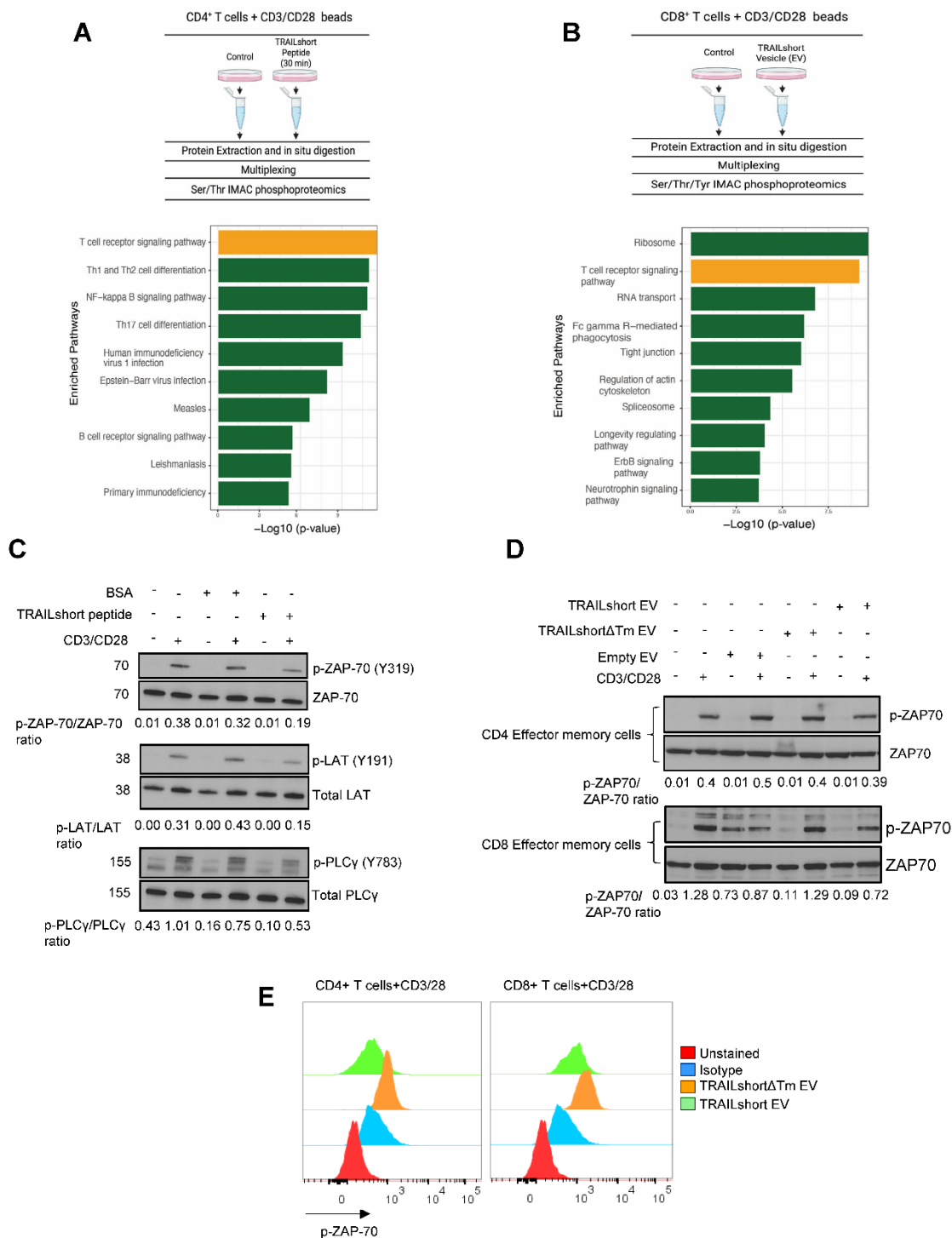
Supplementary Figure Legends

Suppl. Fig. 1



Supplementary Fig. 1. (A) Schematic representation of the generation of GFP-tagged TRAILshort EVs in TRAIL knockout 293T cells. (B) Western blot analysis of the isolated EVs confirms the expression of TRAILshort and TRAILshortΔTm, as detected using an anti-TRAILshort antibody, while HSP70 serves as an EV-specific marker. (C) Western blotting shows the absence of DR5 expression in 293T DR5 knockout cells. (D) Schematic diagram of the experimental setup showing the treatment of wild-type and DR5 knockout 293T cells with GFP-TRAILshort containing EVs pre-labeled with pH-sensitive dye-conjugated antibodies to track EV internalization (left). The representative confocal fluorescence image (right) shows GFP-TRAILshort EV uptake in wild-type 293T cells, visualized as yellow puncta, indicating DR5-dependent EV internalization.

Suppl. Fig. 2



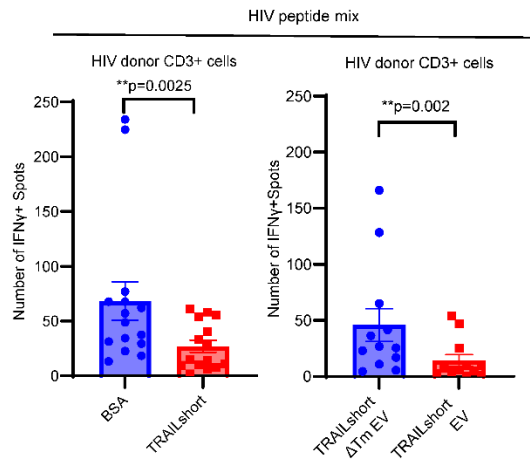
Supplementary Fig. 2.

(A & B) Experimental conditions for CD4⁺ and CD8⁺ T cells treated with TRAILshort in peptide or EV form prior to phospho-proteomic analysis. Bar graphs show differentially expressed phospho-

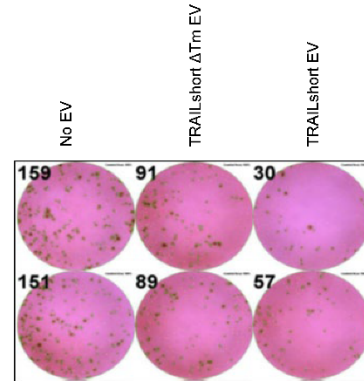
proteins relative to untreated controls. KEGG pathway enrichment was performed on phosphoproteins with $\log_2$ fold change ($\log_2\text{FC}$) > 1.5 or < 0.9 , identifying significant modulation of TCR signaling pathways under both treatment conditions. **(C & D)** Immunoblot analysis of primary CD3⁺ T cells pretreated with BSA or TRAILshort peptide (5 $\mu\text{g/mL}$) **(C)**, or with TRAILshort ΔTm or TRAILshort EVs (1000:1 EV:cell ratio) **(D)** for 2-3 hours, followed by stimulation with CD3/CD28 beads (1:2 bead:cell ratio) for 30 min. Blots were probed for total and phosphorylated ZAP-70, LAT, or PLC γ . Values indicate the ratio of phosphorylated to total protein. **(E)** Histograms showing flow cytometry analysis of p-ZAP-70 in CD4⁺ and CD8⁺ T cells treated with TRAILshort or TRAILshort ΔTm EVs and stimulated with CD3/CD28 beads, as indicated above.

Suppl. Fig. 3

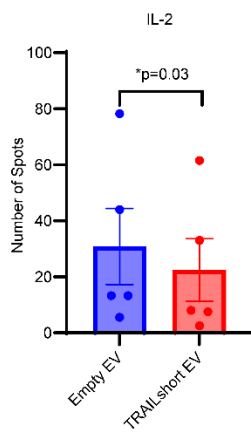
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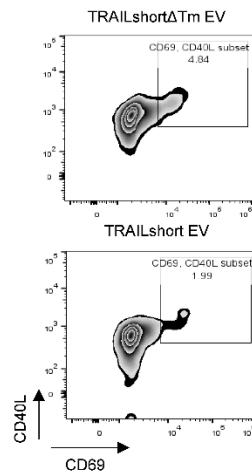
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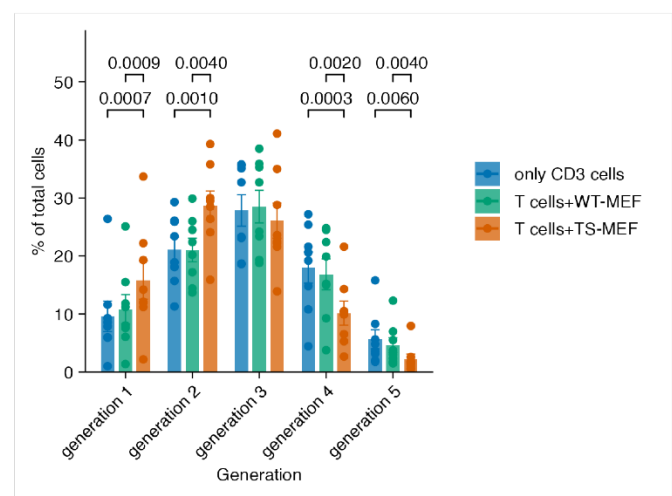
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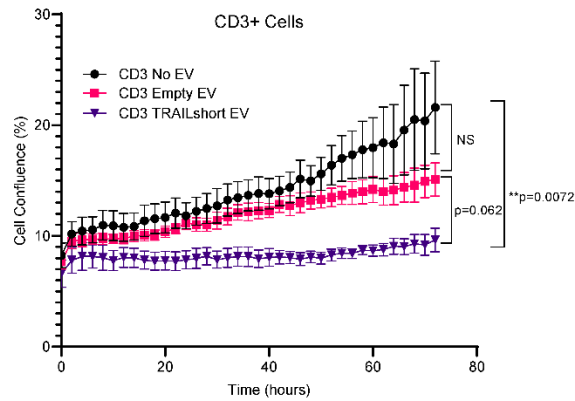
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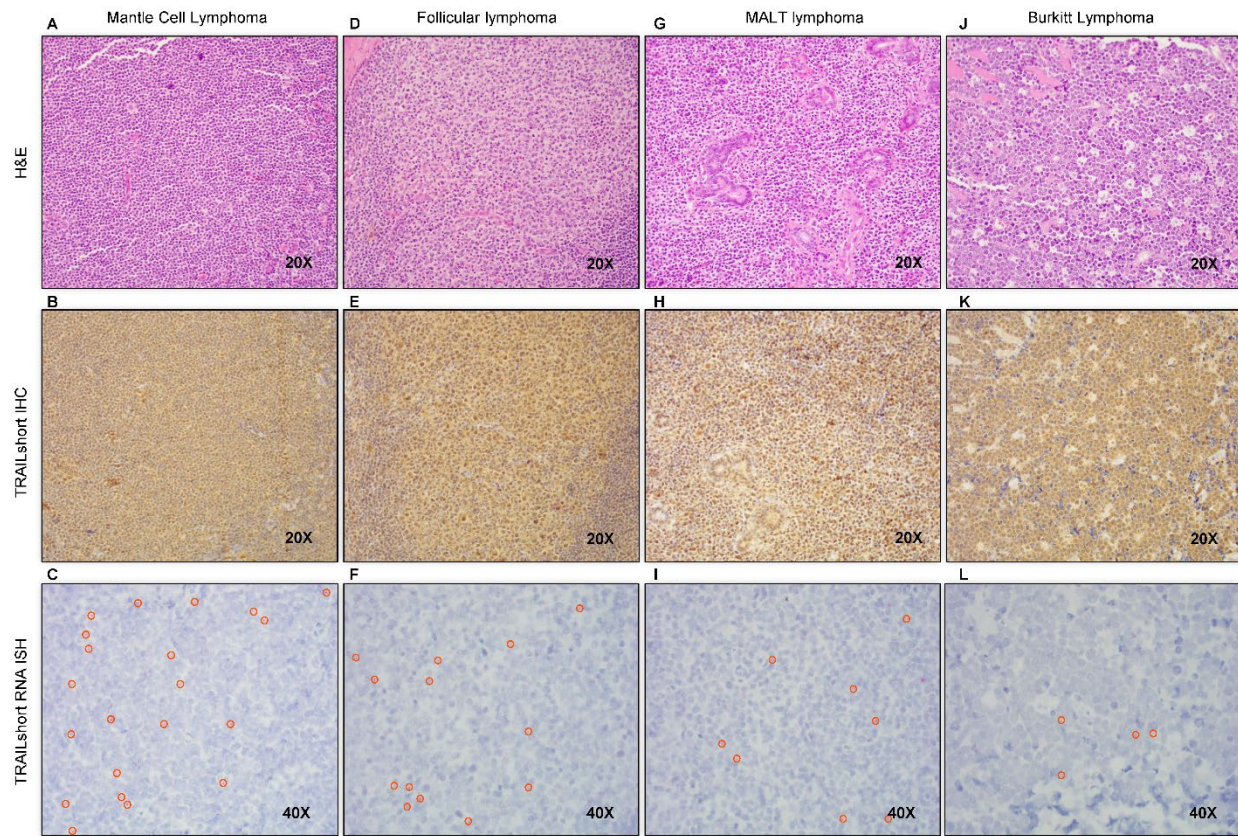


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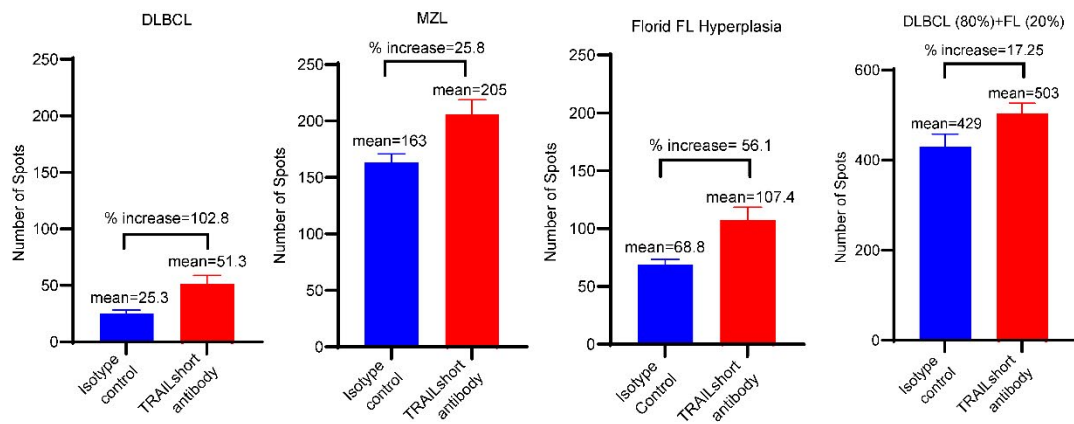


Suppl. Fig. 3

G



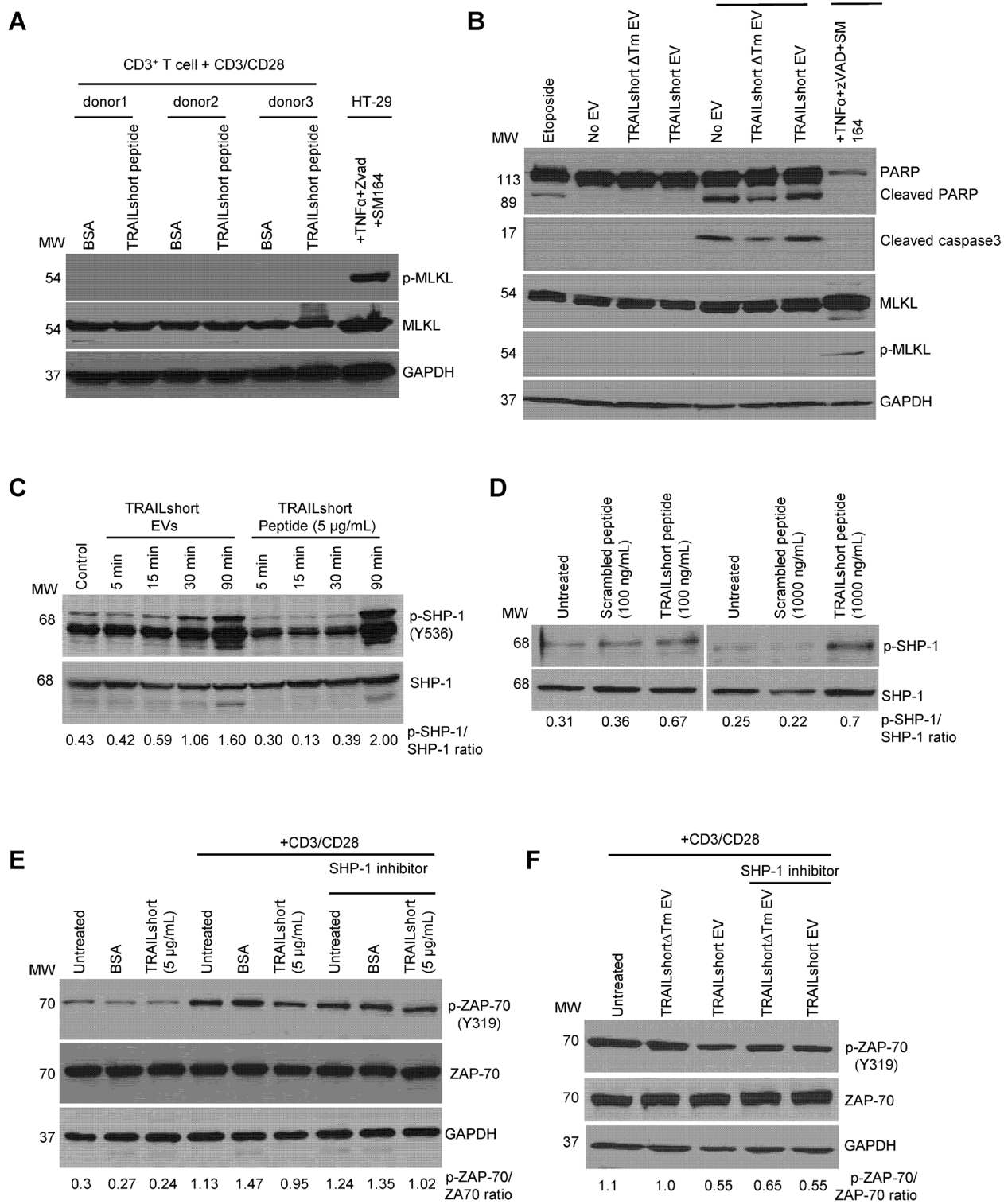
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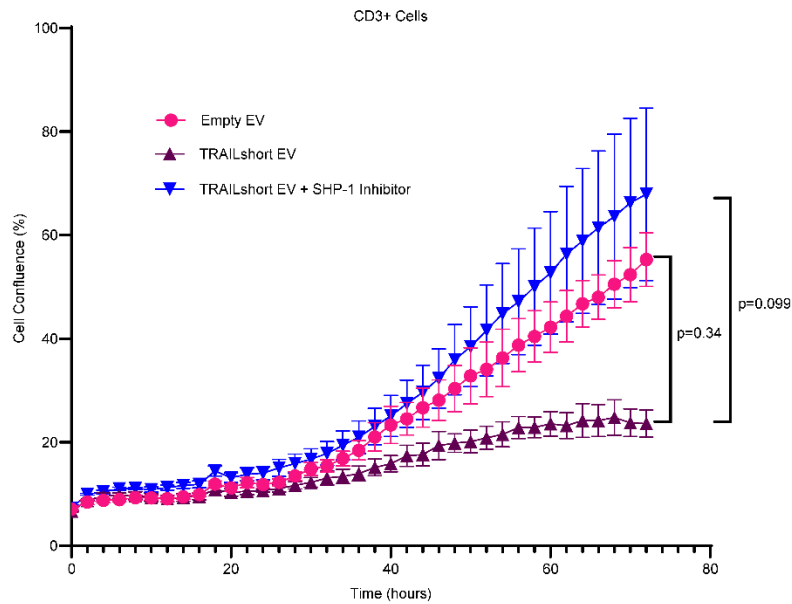
Supplementary Fig. 3.

(A) Bar graph showing ELISpot analysis of IFN γ spot formation in T cells treated with BSA or TRAILshort peptide (5 μ g/mL) or exposed to TRAILshort Δ Tm or TRAILshort EVs (1:1000 cell-to-EV ratio), followed by stimulation with HIV pepmix. Statistical analysis using the Wilcoxon matched-pairs signed-rank test shows a significant difference between TRAILshort-treated samples (EV or peptide) and their corresponding control-treated samples ($p < 0.05$). **(B)** Representative ELISpot images obtained using a CTL reader demonstrating reduced IFN γ spot formation following TRAILshort EV treatment. **(C)** Quantification of IL-2 secretion from T cells treated with TRAILshort EVs and co-cultured with syngeneic macrophages loaded with tetanus antigen, as measured using a Human IL-2 FluoroSpot assay kit. TRAILshort EV treatment significantly reduces IL-2 spot formation compared with control conditions (paired t-test; $p < 0.05$). **(D)** Flow cytometry analysis showing a reduced frequency of activated T cells (CD40L $^+$ /CD69 $^+$) in response to TRAILshort EV exposure. **(E)** Bar graphs showing statistical analysis (paired t-test; $p < 0.05$) of T-cell proliferation assessed by CFSE dilution following treatment with TRAILshort. **(F)** CD3 $^+$ T cells were treated with no EVs, empty vector EVs, or TRAILshort EVs and subsequently stimulated with CD3/CD28 beads overnight. Cell proliferation was monitored for 72 hours using IncuCyte live-cell imaging. Cell growth was quantified by calculating the area under the confluence-versus-time curve (AUC). Mean AUC values from technical replicate wells were compared using ordinary one-way ANOVA followed by Tukey's multiple-comparisons test (** $p < 0.01$). **(G)** Lymphoid tissues from patients with the indicated B-cell malignancies exhibit TRAILshort positivity by immunohistochemistry (IHC) and *in situ* hybridization (ISH): (a–c) Mantle Cell Lymphoma, (d–f) Follicular Lymphoma, (g–i) MALT Lymphoma, and (j–l) Burkitt Lymphoma. **(H)** Bar graphs summarizing IFN γ ELISpot data from T cells isolated from spleens of lymphoma patients. T cells were stimulated with CD3/CD28 beads and co-cultured with autologous B-cell tumors in the presence of either anti-TRAILshort antibody or matched isotype control. Patient cohorts included Chronic Lymphocytic Leukemia (CLL), Follicular Lymphoma (FL), and Diffuse Large B-cell Lymphoma (DLBCL). TRAILshort antibody increases the number of IFN- γ spots.

Suppl. Fig. 4



Suppl. Fig. 4G

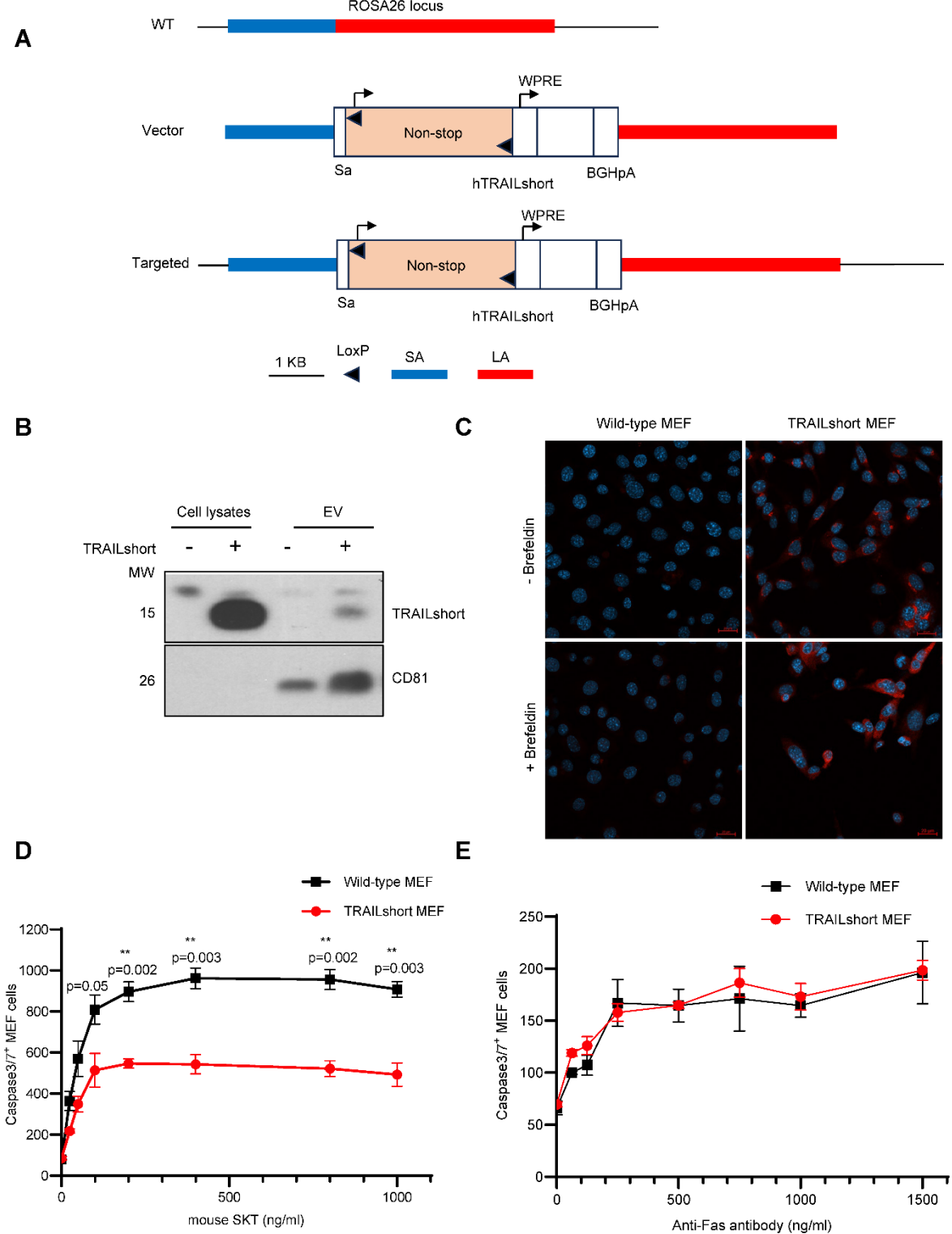


Supplementary Fig. 4.

(A) Primary CD3⁺ T cells were treated with TRAILshort (5 μ g/mL) or BSA control and stimulated with CD3/CD28 beads (1:2 bead-to-cell ratio). After 48 h, cell lysates were analyzed by immunoblotting for phosphorylated MLKL (p-MLKL) and total MLKL to assess necroptosis. **(B)** CD3⁺ T cells were treated with TRAILshort Δ Tm or TRAILshort-containing EVs at a 1:1000 cell-to-EV ratio. Lysates were immunoblotted for cleaved caspase-3 and cleaved PARP (apoptosis markers), as well as p-MLKL and total MLKL (necroptosis markers). GAPDH served as a loading control. HT-29 cells treated with TNF α (20 ng/mL), zVAD (20 μ M), and SM164 (100 nM), and CD3⁺ T cells treated with etoposide (10 μ M), were used as positive controls. MLKL, mixed lineage kinase domain-like protein; PARP, poly (ADP-ribose) polymerase. **(C)** Primary CD3⁺ T cells were treated with TRAILshort-containing EVs or TRAILshort peptide (5 μ g/mL) for the indicated times, followed by immunoblot analysis of total and phosphorylated SHP-1. **(D)** Cells were treated with scrambled or TRAILshort peptide (100 or 1000 ng/mL) for 30 min and analyzed by immunoblotting for total and phosphorylated SHP-1. **(E, F)** Primary CD3⁺ T cells were pretreated with 1 μ M SHP-1 inhibitor (NSC-87877) for 1 h, then exposed to either TRAILshort Δ Tm or TRAILshort EVs **(E)**, or to BSA or TRAILshort peptide (5 μ g/mL) for 1 h **(F)**, prior to stimulation with CD3/CD28 beads (1:1 bead-to-cell ratio). Lysates were immunoblotted for total and phosphorylated ZAP70, with GAPDH as a loading control. **(G)** CD3⁺ T cells were treated with TRAILshort EV or TRAILshort

EV plus an SHP-1 inhibitor (0.5 μ M), followed by overnight stimulation with CD3/CD28 beads. T cell proliferation was monitored using IncuCyte Live-Cell analysis over 72 hours. Mean AUC values from technical replicate wells were compared using ordinary one-way ANOVA followed by Tukey's multiple-comparisons test.

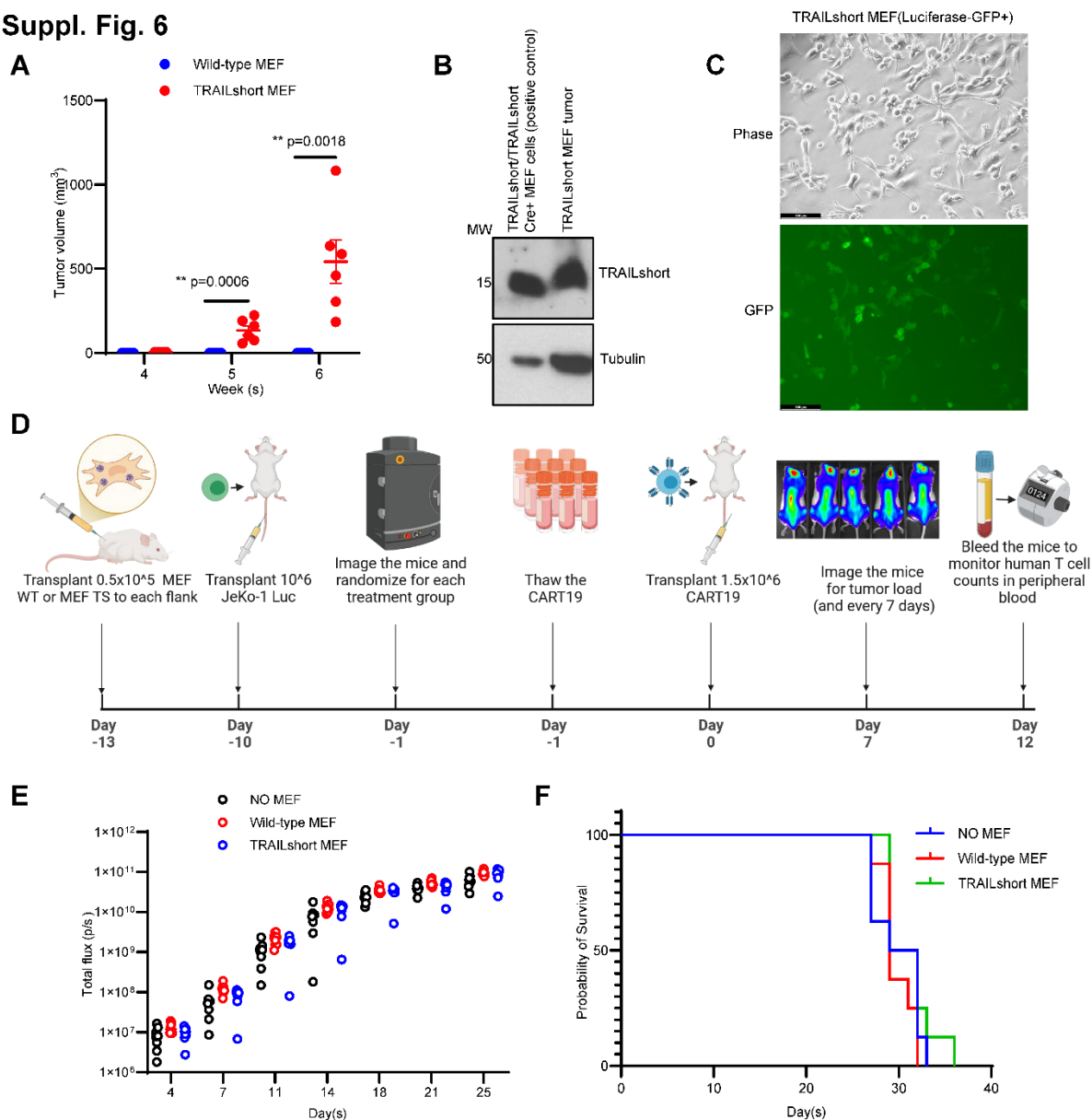
Suppl. Fig. 5



Supplementary Fig. 5.

(A) Schematic representation of the strategy used to generate ROSA26 transgenic human TRAILshort knock-in mice. **(B)** Western blot analysis showing that TRAILshort is expressed in mouse embryonic fibroblasts (MEFs) isolated from TRAILshort knock-in mice and is also secreted in the EVs released by these MEFs. Blots were probed using an anti-TRAILshort antibody (mouse version; 2.2). CD81 is an EV marker. **(C)** Immunofluorescence staining reveals TRAILshort protein expression in TRAILshort MEF cells, but not in wild-type MEF cells. Brefeldin A treatment leads to the accumulation of TRAILshort in the cytosol of TRAILshort MEF cells. **(D & E)** MEFs expressing TRAILshort are resistant to apoptosis induced by mouse super-killer TRAIL (mouse-SKT) but remain susceptible to Fas-mediated cell apoptosis. Wild-type and TRAILshort MEFs were cultured with increasing doses of mouse SKT **(D)** or an anti-Fas antibody **(E)**, and apoptosis was assessed by quantifying positive cells for active caspase-3/7 staining using IncuCyte over a 40-hour period. The graphs show IncuCyte data collected at the 30-hour time point post-treatment. Two-sample t-test shows significant differences between wild-type and TRAILshort MEFs at the indicated concentrations (**p <0.01).

Suppl. Fig. 6

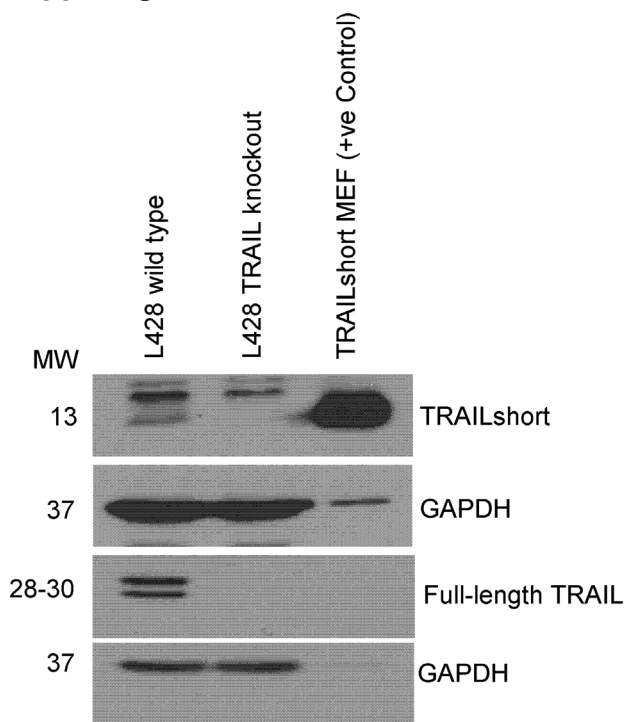


Supplementary Fig. 6.

(A) Graphs showing tumor volume of palpable tumors detected 4 weeks after wild-type or TRAILshort MEF cell transplantation in CD34⁺ humanized mice. Multiple t-tests with Bonferroni correction were used to compare tumor volume between two animal groups. **p<0.01 denotes significant difference between groups. **(B)** Tumor tissues were dissected from mice, minced, lysed and run on SDS-PAGE gel. Western Blot shows the expression of TRAILshort in the tumors derived from TRAILshort MEFs in CD34⁺ humanized mice. **(C)** Cell suspensions isolated from dissected TRAILshort MEF tumors were cultured and analyzed for GFP expression using fluorescent microscope (400X magnification, Scale bar=100 µm). **(D)** Diagram illustrating the

detailed procedure for implanting MEF (wild-type and TRAILshort MEF) and JeKo-1 cells, followed by the introduction of CAR-T19 cells into NSG mice. **(E)** Mice were subcutaneously implanted in both flanks with 5×10^5 wild-type MEFs ($n = 8$), TRAILshort-expressing MEFs lacking luciferase constructs ($n = 8$), or PBS (no MEF control; $n = 8$). Three days later, 1×10^6 luciferase-labeled JeKo-1 cells were injected intravenously. Tumor engraftment and progression were tracked for 25 days by bioluminescence imaging (IVIS). No significant differences in tumor growth were observed among groups. **(F)** Kaplan–Meier survival analysis showing no significant differences in survival across mice corresponding to the experimental groups described in **(E)**.

Suppl. Fig. 7



Supplementary Fig. 7.

Western blot analysis shows the levels of full-length TRAIL and TRAILshort protein expression, in both wild-type and TRAIL knockout L428 cells, with GAPDH used as a loading control. TRAILshort expressing MEF cells are used as positive control.