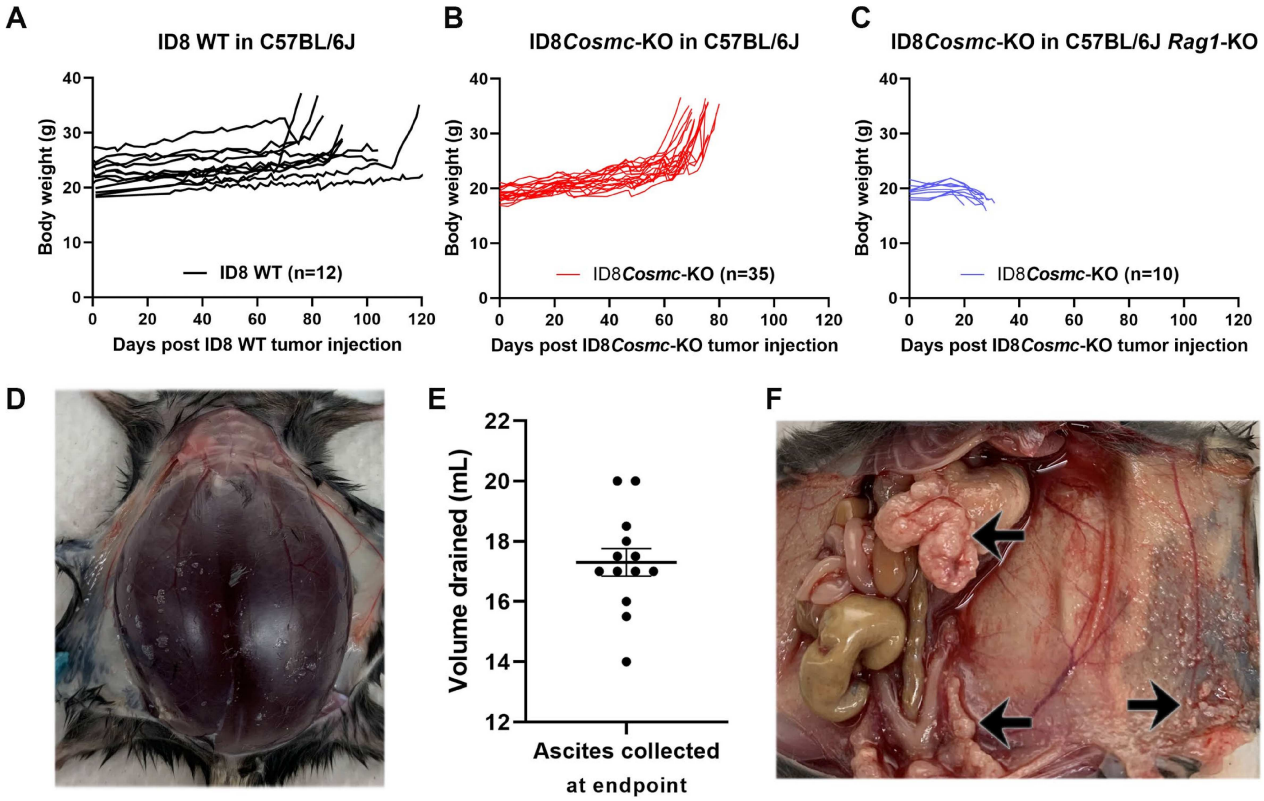
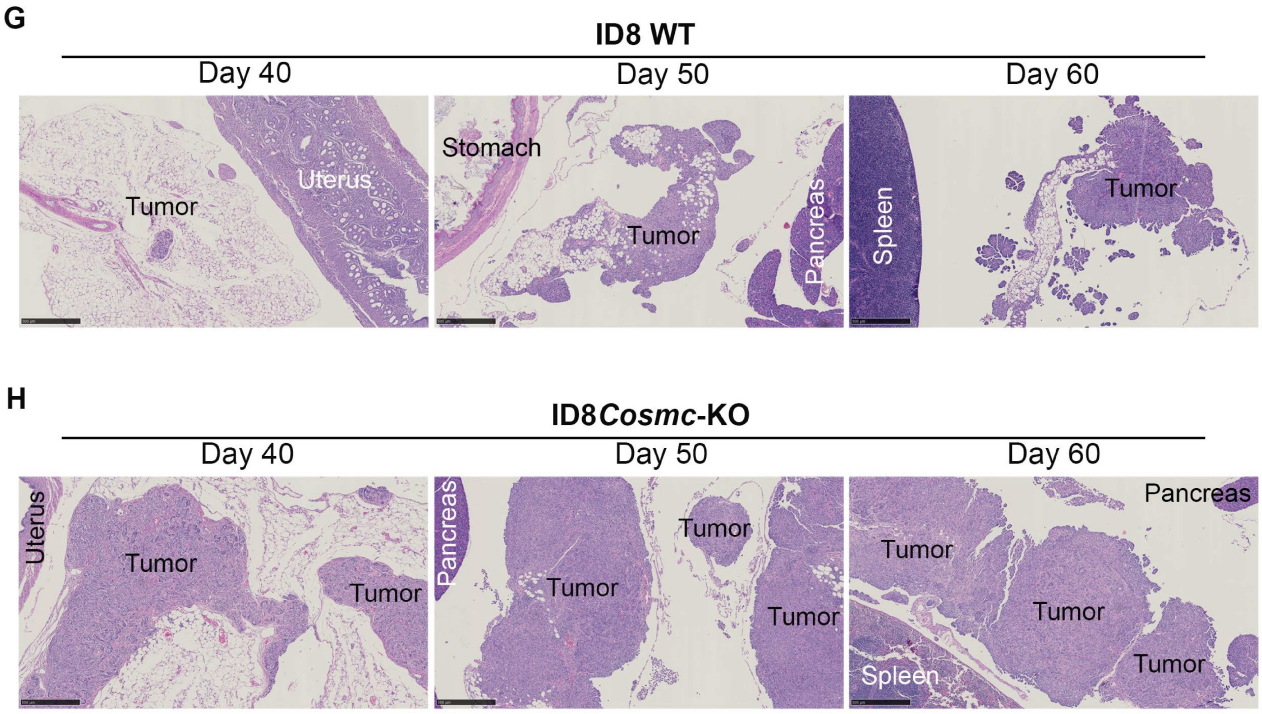
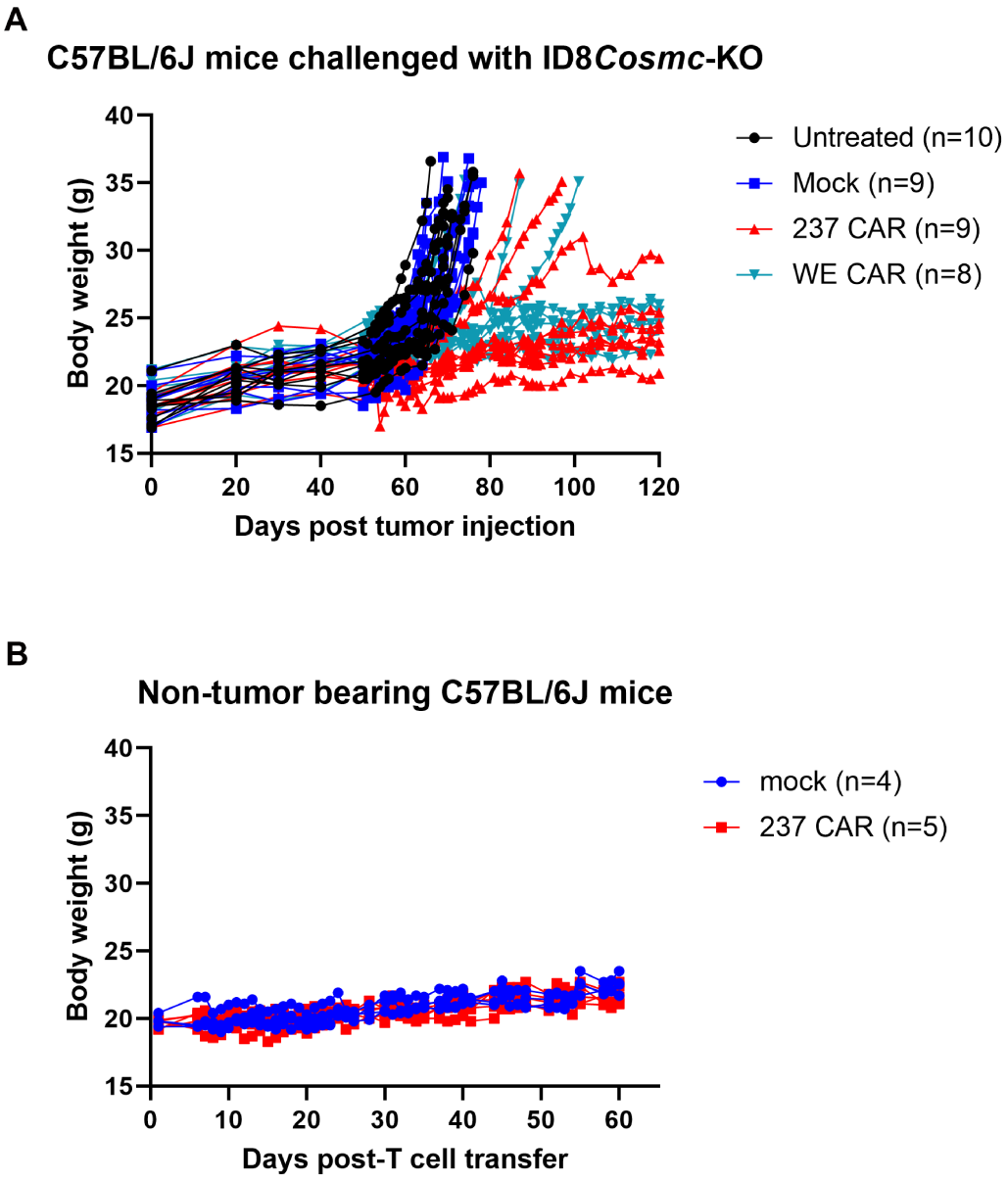
Supplemental Figure S1





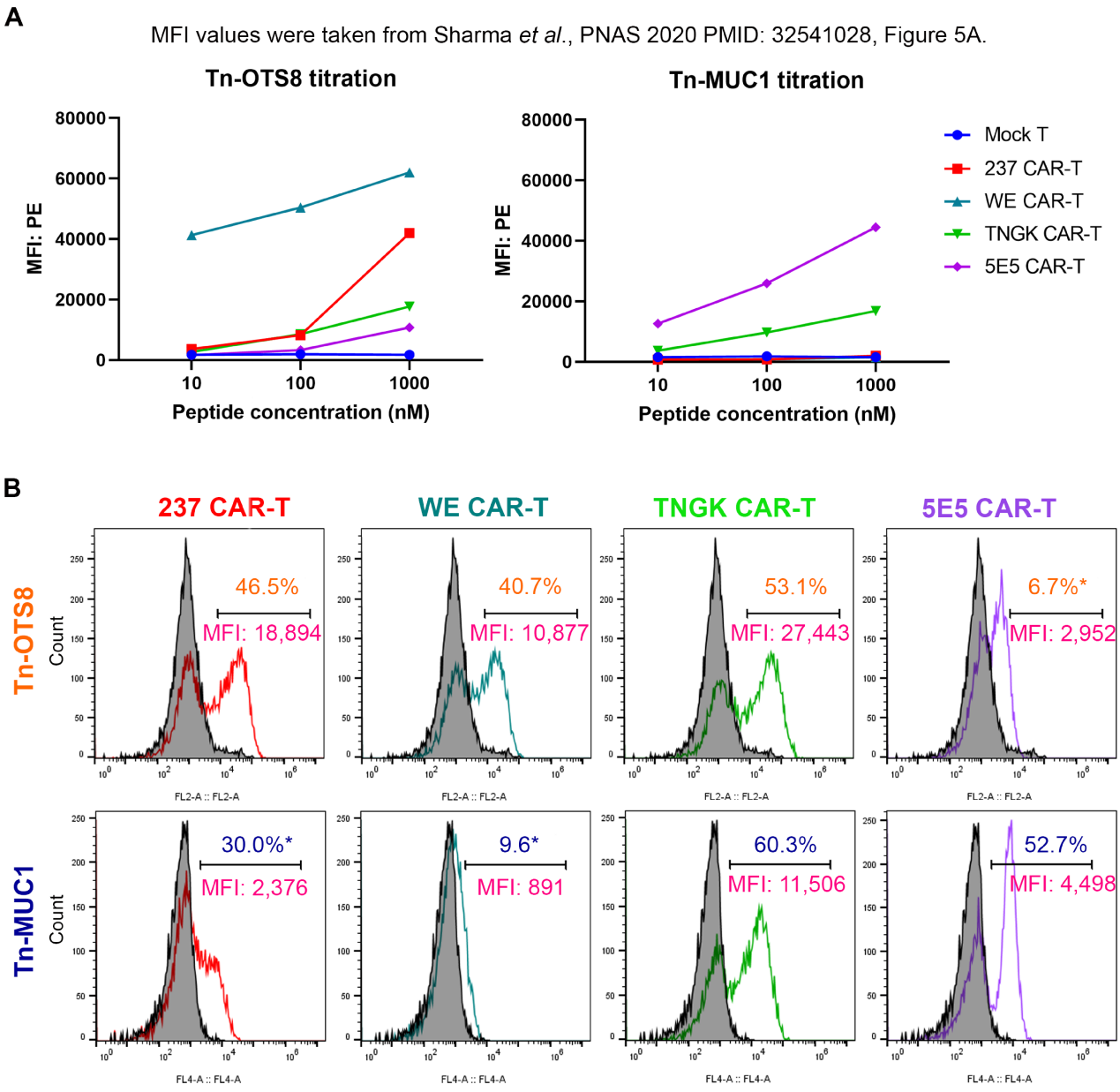
Supplemental Figure S1. Characterization of the ID8Cosmc-KO tumor model. (**A, B**) Body weights over time of untreated C57BL/6J mice inoculated with either ID8 WT (**A**) or ID8*Cosmc*-KO (**B**) via i.p. injection. Figures were plotted with GraphPad Prism v9.4.1 using cumulative data across multiple experiments with a total n=12 or n=35 for ID8 WT and ID8*Cosmc*-KO untreated cohorts, respectively. (**C**) Body weights of C57BL/6J *Rag1*-KO mice inoculated with ID8*Cosmc*-KO (n=10). (**D**) Representative image of an ID8Cosmc-KO-inoculated C57BL/6J mouse at endpoint criterion. The epidermal skin layer was carefully removed to visualize the distended abdomen filled with ascites fluid. (**E**) An average of 17mL ascites fluid was drained from mice that reached endpoint criterion. Dot plot was generated using GraphPad Prism v9.4.1. Each dot represents one mouse and error bars are SEM. (**F**) Representative image of an ID8*Cosmc*-KO-inoculated C57BL/6J mouse at endpoint criterion after incision of the muscle layer, revealing the extensive tumor nodules (black arrows) disseminated throughout the intraperitoneal space and the omentum. (**G**-**H**) Tissue sections from ID8 WT and ID8*Cosmc*-KO mice at various times after tumor challenge. Formalin-fixed paraffin embedded tissue sections from the peritoneal cavity of mice inoculated with either ID8 WT (**G**) or ID8*Cosmc*-KO (**H**) cells at the indicated time points were stained with H&E. Tumor foci and neighboring organs are indicated. Scale bar = 500μm

Supplemental Figure S2



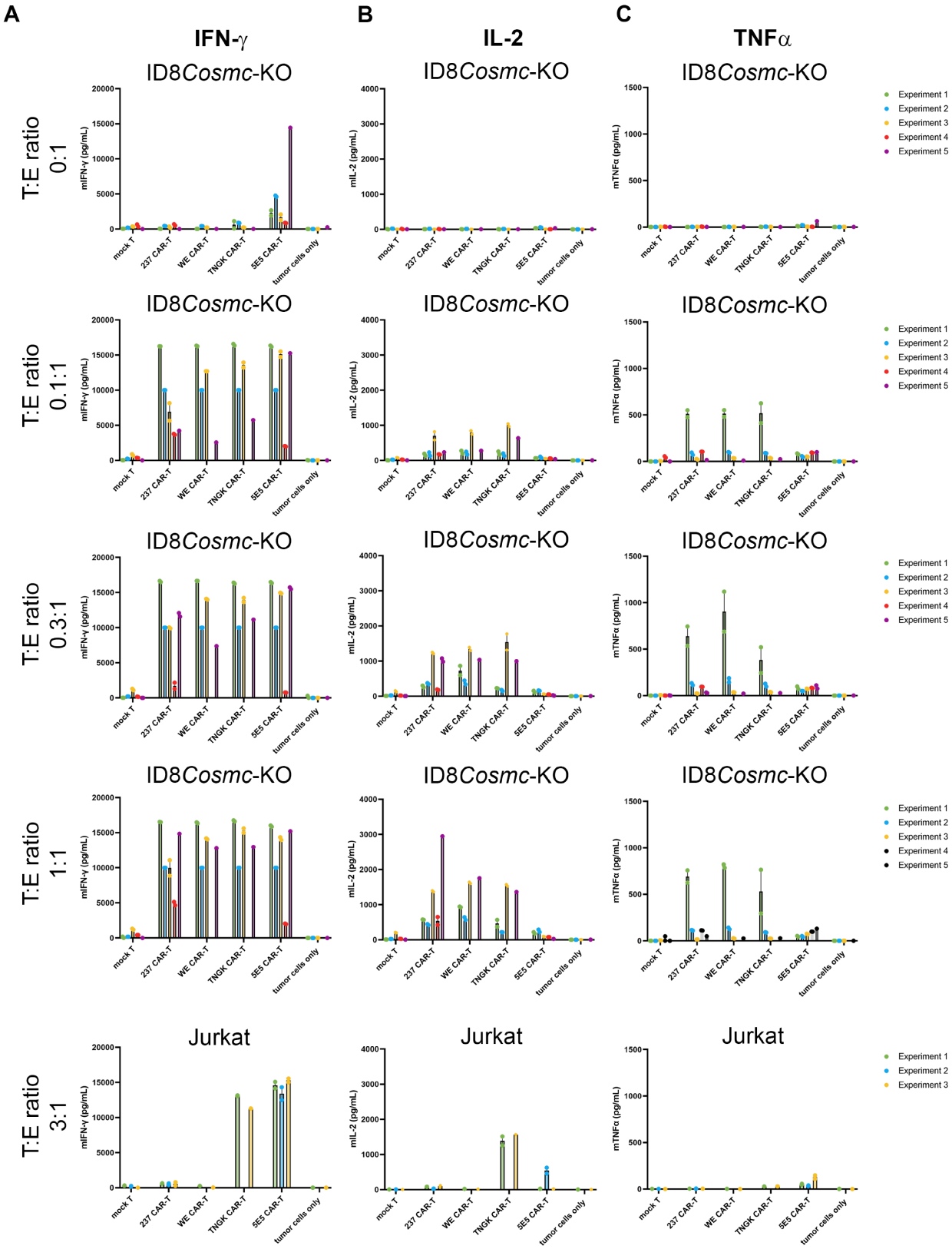
Supplemental Figure S2. Weights of C57BL/6J mice administered 237 CAR T in tumor-bearing (**A**) and non-tumor bearing (**B**) C57BL/6J mice. (**A**) ID8*Cosmc*-KO-innoculated mice were administered 5x10^6 237 or WE CAR-T cells (or mock-transduced T cells) at day 50 via i.p. injection. (**B**) Non-tumor bearing mice were administered 5x10^6 237 CAR-T cells (or mock-transduced T cells) by i.p. injection. Body weights were measured over a period of 60 days post-T cell transfer. Line graphs were plotted using GraphPad Prism v9.4.1. Each line represents 1 mouse.

Supplemental Figure S3

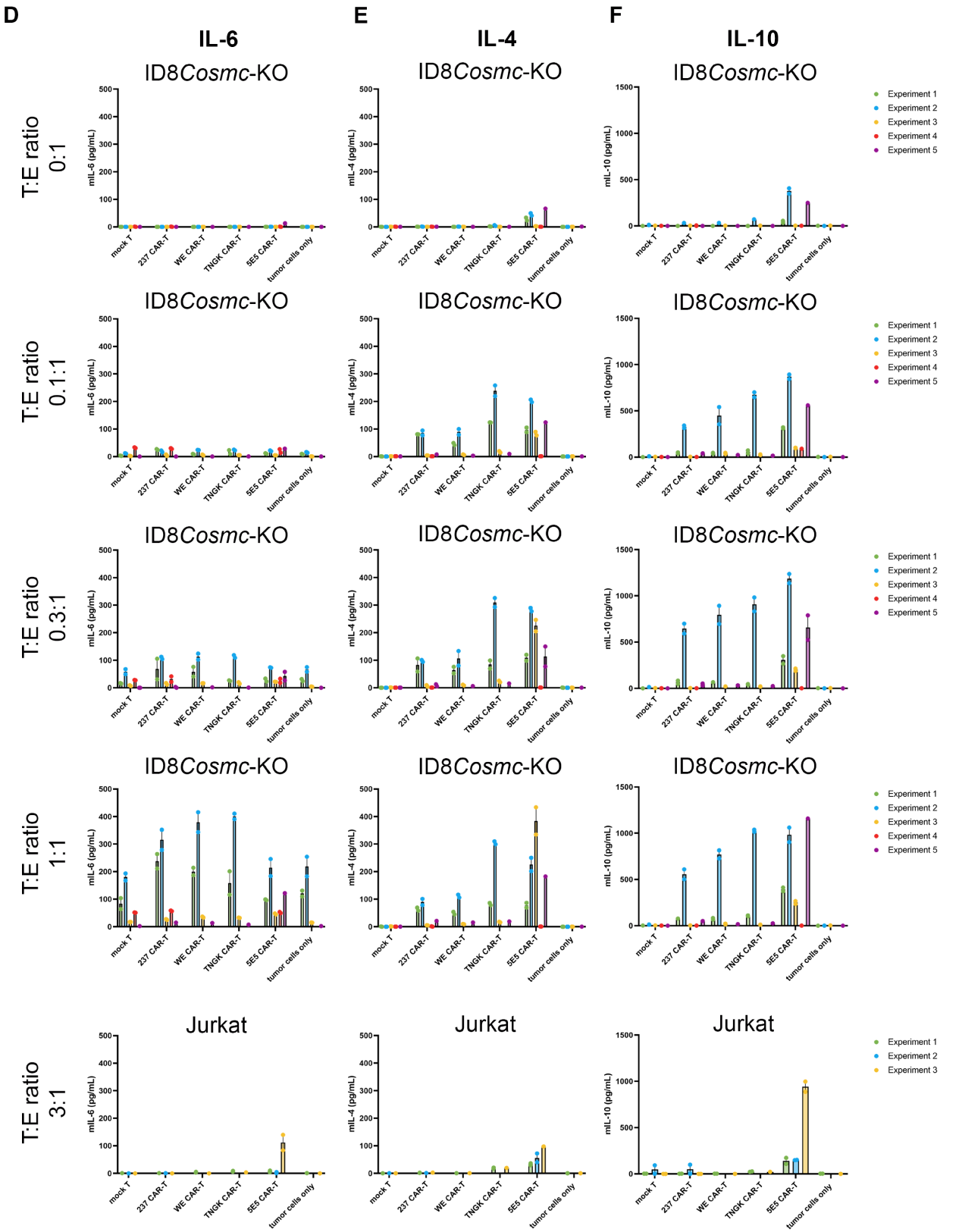


Supplemental Figure S3. Binding of biotinylated Tn-peptides to the four CAR-T cells. (**A**) Mock, 237, WE, TNGK, and 5E5 CAR-T cells were stained with various concentration of biotinylated Tn-OTS8 peptide (left) or Tn-MUC1 peptide (right), followed by streptavidin-PE. Mean fluorescent intensity (MFI) plotted against peptide concentration illustrate the affinity of CAR-T cells for their respective peptide Tn-antigens. MFI values were taken from Sharma et al., PNAS 2020 PMID: 32541028. Plots shown are representative of two independent experiments. (**B**) Binding of 237, WE, TNGK, and 5E5 CAR-Ts to either 10nM biotinylated Tn-OTS8 tetramer prepared with streptavidin-PE (top row) or 100nM biotinylated Tn-MUC1 followed by streptavidin-Alexafluor647 (bottom row). Flow cytometry plots were overlaid against mock-transduced T cells (shaded in gray). The percentage of positive peaks and their MFI are indicated for each plot.

Supplemental Figure S4

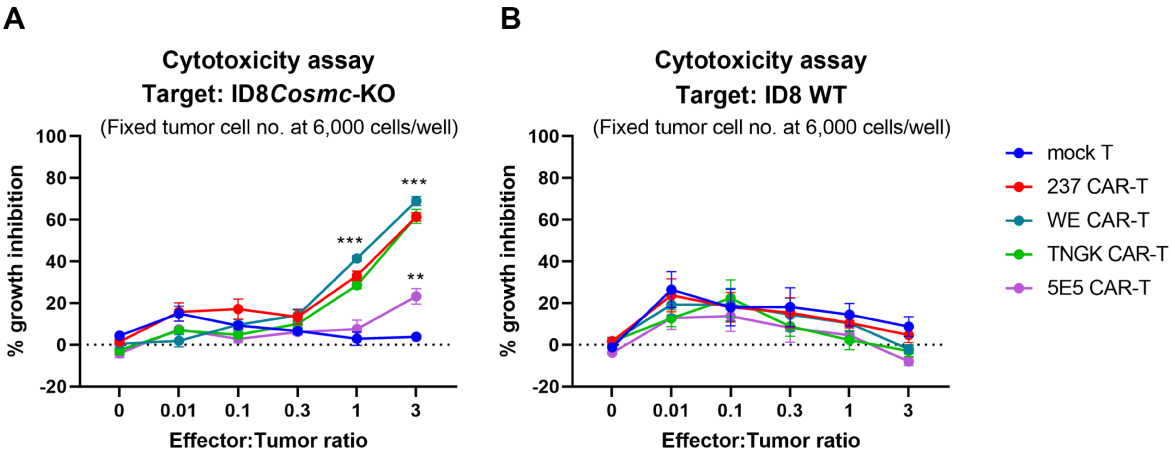


Supplemental Figure S4, cont.



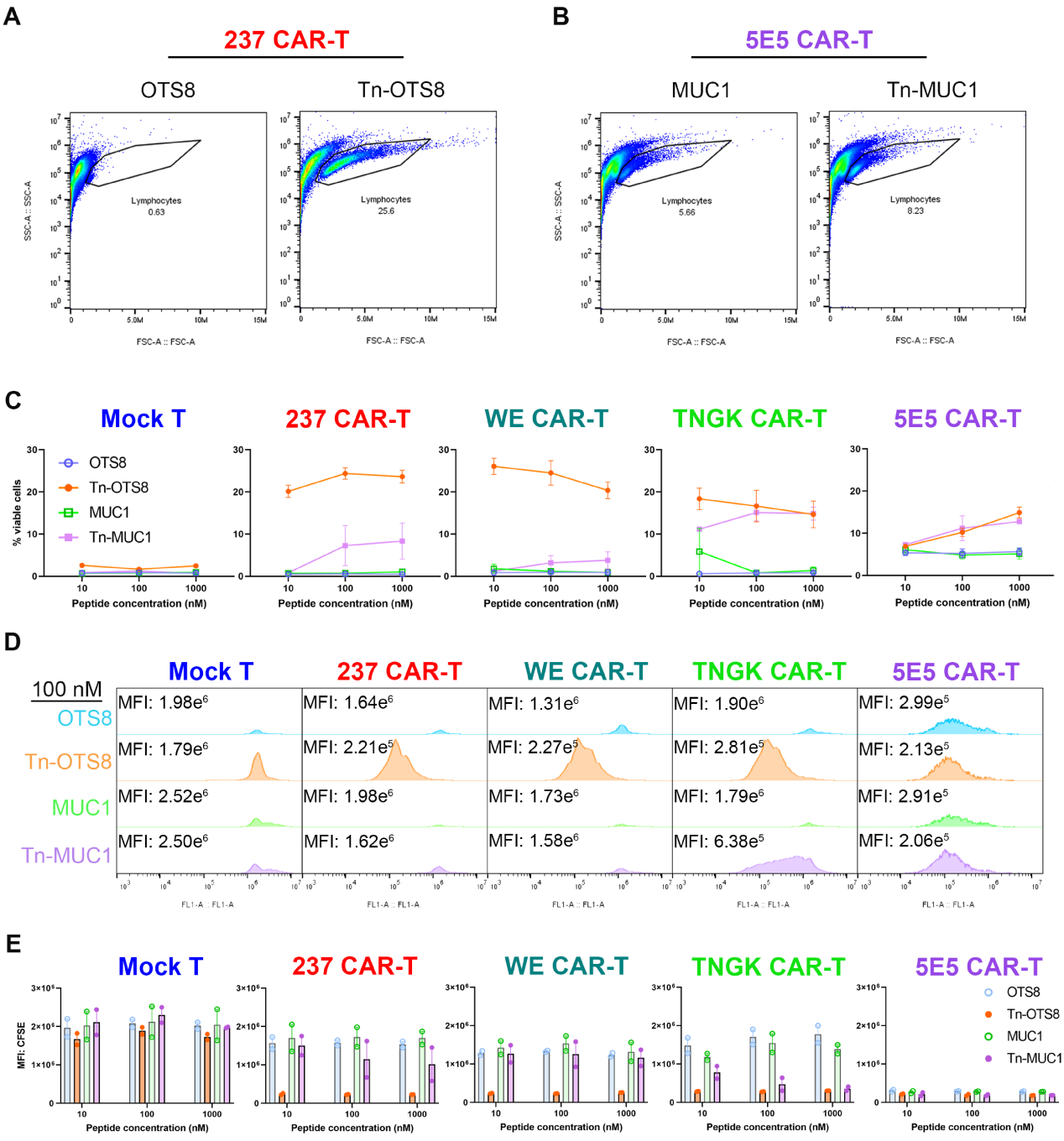
Supplemental Figure S4. In vitro cytokine analyses of CAR-T cells co-cultured with ID8Cosmc-KO or Jurkat at the indicated tumor:effector ratios. Cell culture supernatant from mock, 237-CAR, WE-CAR, TNGK-CAR, or 5E5-CAR transduced primary T cells were co-cultured with ID8*Cosmc*-KO cells in 96 well plates and supernatants were analyzed after 24 hours for cytokines IFN-γ (**A**), IL-2 (**B**), TNFα (**C**), IL-6 (**D**), IL-4 (**E**), and IL-10 (**F**) using a cytokine array kit read in a Luminex 200 plate reader. The tumor to effector (T:E) ratio is indicated in each row. A total of five independent experiments were performed, represented by each bar in the plots. Bottom row plots correspond to data obtained from co-culture with Jurkat cells at a 3:1 T:E ratio, and results from three independent experiments are shown. Statistically significant comparisons between 237 and 5E5 from all five experiments are shown in Figure 5.

Supplemental Figure S5



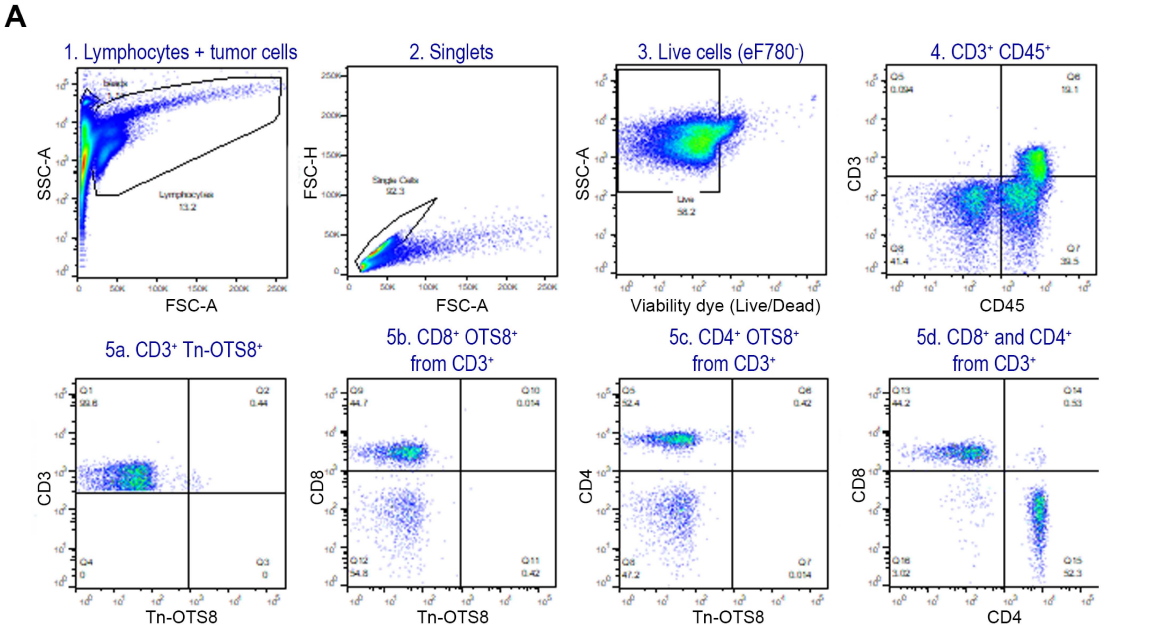
Supplemental Figure S5. Comparison of 237, WE, TNGK and 5E5 CAR T cells in killing assays in vitro (growth inhibition). ID8*Cosmc*-KO (**A**) and ID8 WT (**B**) were examined with mock, 237-CAR, WE-CAR, TNGK-CAR, or 5E5-CAR transduced primary T cells from C57BL/6J mice, co-cultured with either ID8*Cosmc*-KO or ID8 WT cells in a 96-well plate for 24 hours at various tumor:effector ratios. The number of tumor cells were kept constant at 6,000 cells/well across all conditions tested. After 24 hours, culture supernatants were removed and the adherent cells were fixed in ice-cold 10% trichloroacetic acid for the SRB assay. The % growth inhibition was calculated using the formula outlined in Methods section. This experiment was performed twice, each with a technical replicate of n=4. Representative line graphs were plotted using GraphPad Prism v9.4.1. Error bars are SEM. Error bars are SEM. P-values for 237, WE, TNGK were calculated against either mock or 5E5 at <0.0001 (or denoted by ***); P-values for 5E5 against either mock T or TNGK are at <0.001 (**).

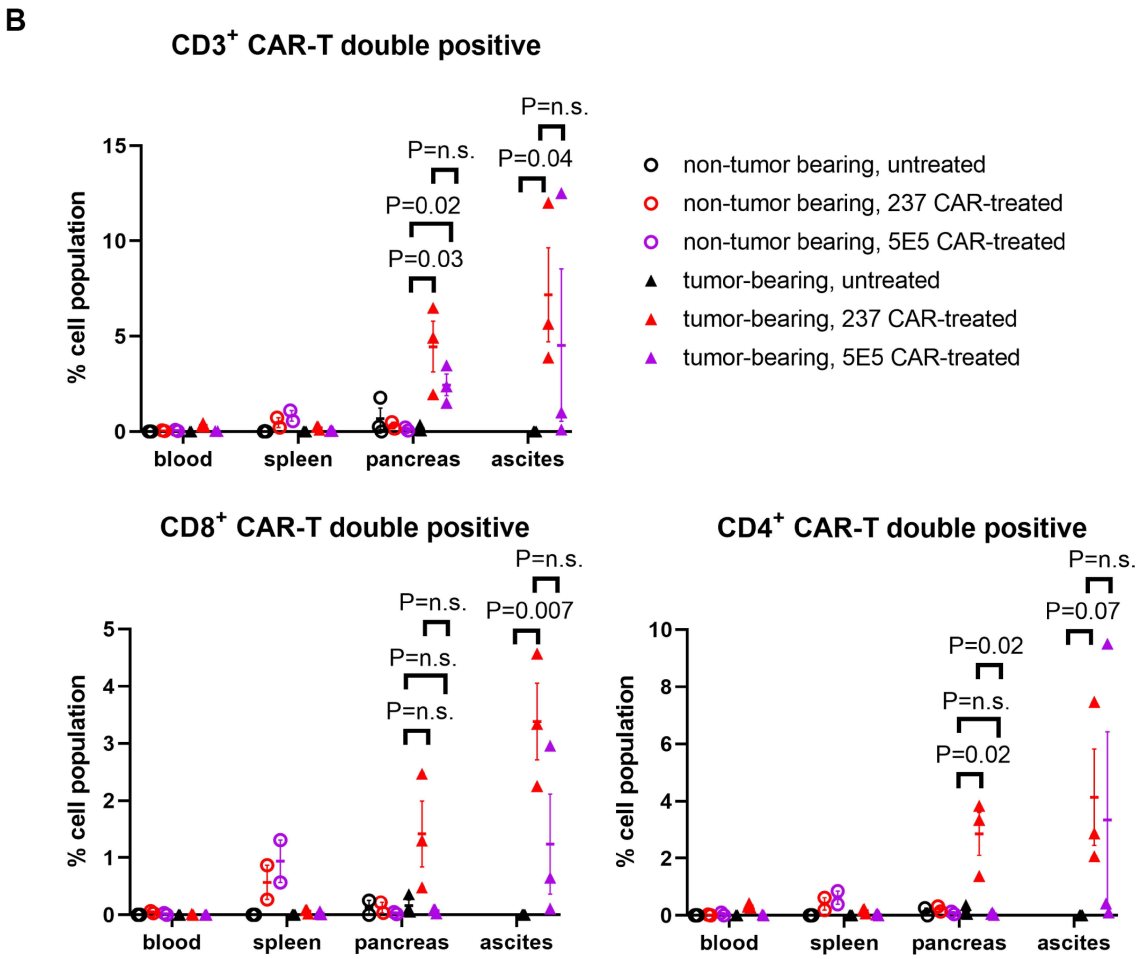
Supplemental Figure S6



Supplemental Figure S6. Antigen-specific proliferation of CFSE-labeled CAR-T cells. Mock, 237, WE, TNGK, 5E5 CAR-Ts were stimulated with various concentrations of OTS8, Tn-OTS8, MUC1, Tn-MUC1 peptides coated on streptavidin immobilized plates. After 72 hours, cells were collected and analyzed by forward versus side scatter gating on the size of lymphocytes. (**A**) Representative images of forward versus side scatter gating of 237 CAR-T cells stimulated with either OTS8 or Tn-OTS8 peptide (**A**) and 5E5 CAR-T stimulated with either MUC-1 or Tn-MUC1 peptide (**B**). (**C**) The percentage of viable T cells in the forward/side scatter gate were plotted against the various concentration of (Tn)-peptides used for stimulation. (**D**) CFSE fluorescence of cells collected for scatter were also analyzed to determine the level of proliferation of viable cells. Flow histograms with indicated MFI for each sample are shown. (**E**) Bar graphs of MFI from CFSE histograms in (D) are shown. Duplicate samples from one experiment were plotted. Lower MFI reflects greater extentof proliferation. Representative of 3 independent experiments, each performed in duplicate.

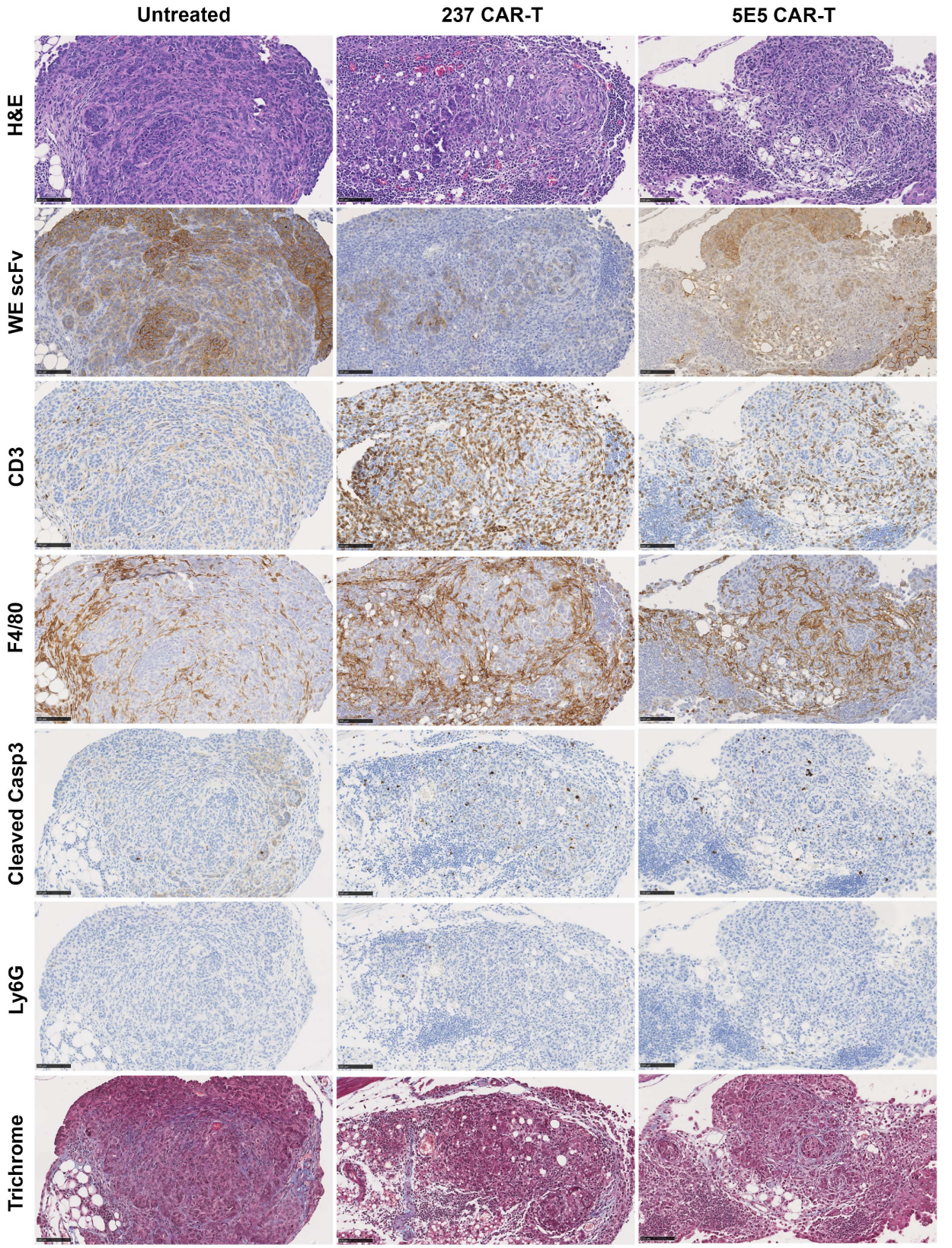
Supplemental Figure S7





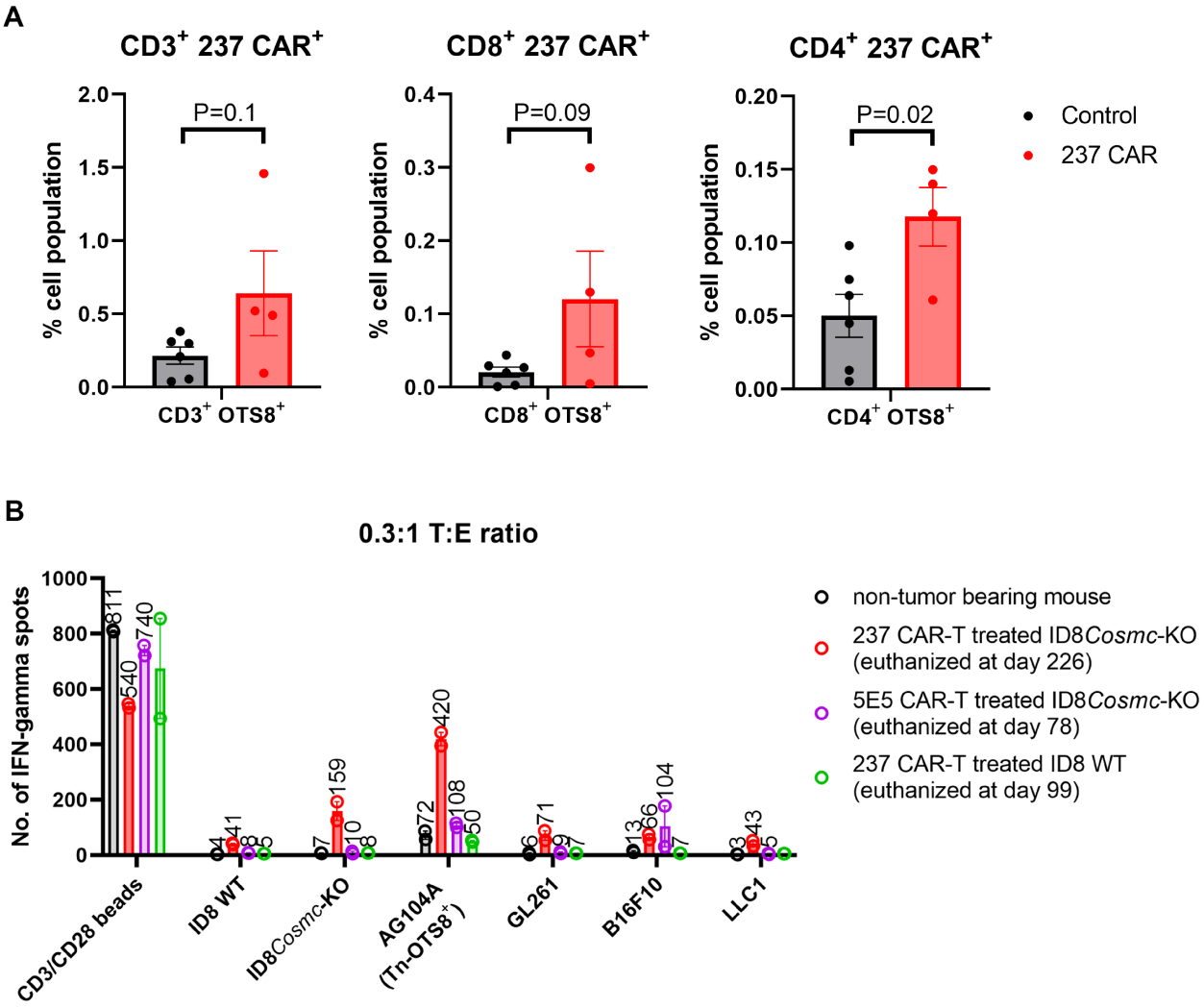
Supplemental Figure S7. Immune cell infiltration in tumors of CAR-treated mice. (**A**) FlowJo gating strategy used for analysis of blood, spleen, pancreas with tumor nodules, and ascites immunophenotyping. The series of gating strategies was as follows: 1) gate on lymphocytes and tumor cells; 2) singlets; 3) live cells; 4) from the population of live cells, gate on CD3+CD45+ double positive cells; and 5) the population of Tn-OTS8+ as well as CD8+ and CD4+ T cells may be derived from the CD3+CD45+ population. (**B**) Immunophenotyping of blood, spleen, tumor nodules in pancreas, and ascites fluid from ID8*Cosmc*-KO-bearing C57BL/6J mice, three days post-treatment with either 237 or 5E5 CAR-T ($5x10^6$ cells) i.p. CAR T cells were administered at day 54 post-tumor challenge. Age-matched non-tumor bearing mice were included as controls, in addition to an untreated ID8*Cosmc*-KO inoculated cohort. Dissociated cells were stained with a cocktail containing anti-CD45, anti-CD3, anti-CD8, and anti-CD4 antibodies and a viability dye. The presence of 237 CAR T in circulation and in tumors was measured in using tetramers of biotinylated Tn-OTS8 peptide included in the antibody cocktail used to stain dissociated cells from tissue samples. Percent cell populations of CD3+, CD8+, and CD4+ T cells were calculated from flow cytometry data obtained using BD LSRII and analyzed using FlowJo software, with n=3 for each tumor-bearing group or n=2 for non-tumor bearing group treated with CAR-Ts. Dot plots were generated using GraphPad Prism v9.4.1. Error bars are SEM.

Supplemental Figure S8



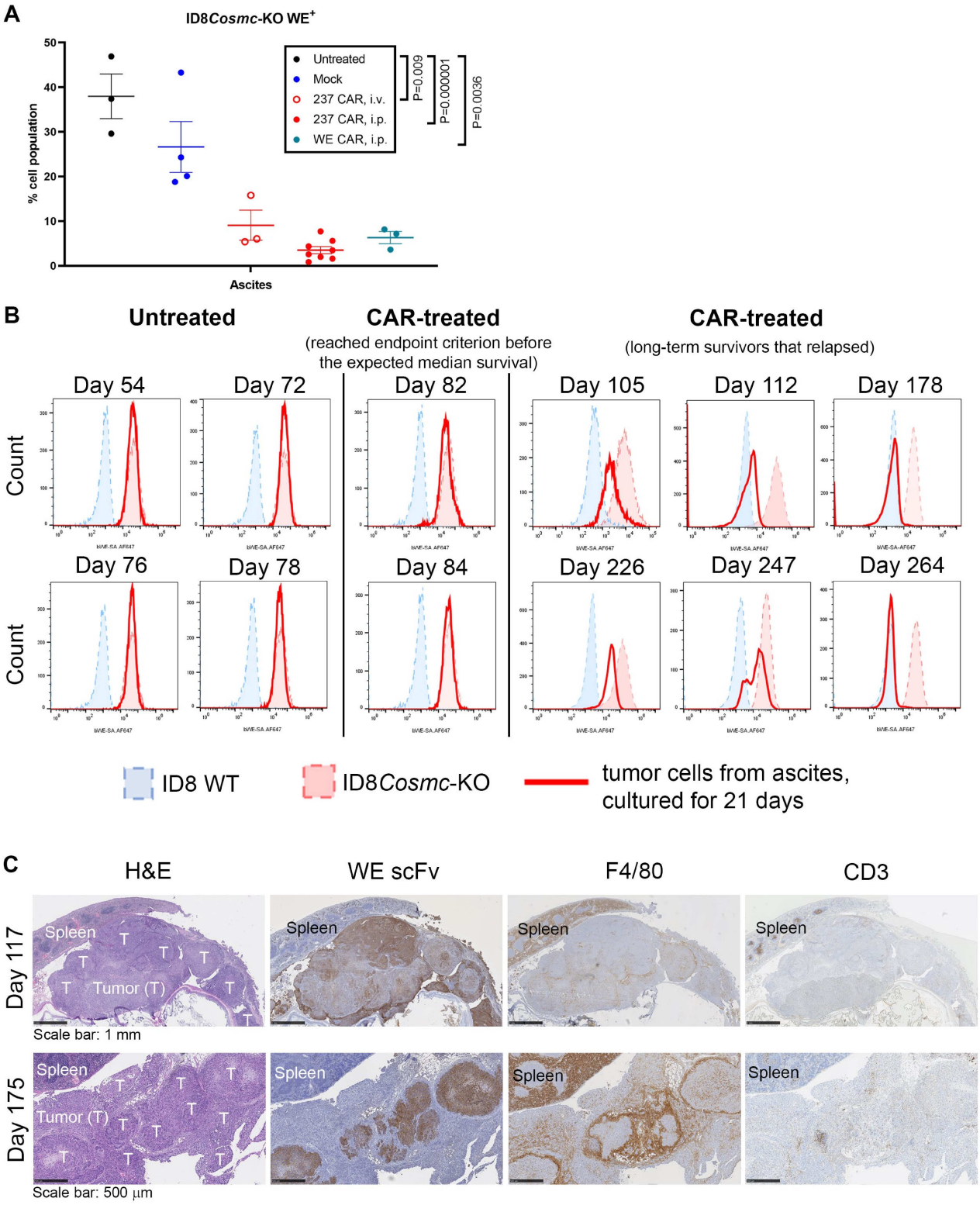
Supplemental Figure S8. Histochemical analysis of tumors from untreated mice or mice treated with 237 or 5E5 CAR-T cells. Peritoneal organs were harvested from ID8*Cosmc*-KO innoculated mice three days after treatment with either 237 or 5E5 CAR-T via i.p. injection at Day 54 post-tumor challenge. Untreated mice were used as control. Immunohistochemistry was performed to visualize Tn-OTS8-glycopeptide$^+$ tumors, CD3$^+$ T cells, F4/80$^+$ macrophages, apoptotic cells, and neutrophils from FFPE tissue sections stained with biotinylated WE-scFv, anti-CD3, anti-F4/80, anti-cleaved Caspase 3, and anti-Ly6G antibodies, respectively. H&E and trichrome stained tissue sections were included as additional reference. Scale bar = 100μm. Quantitation of multiple sections for CD3$^+$ cells, macrophages (anti-F4/80), and apoptotic cells (anti-cleaved Caspase 3) are shown in Figure 6.

Supplemental Figure S9



Supplemental Figure S9. Evidence for in vivo persistence of 237 CAR T cells. (**A**) Blood samples were collected at 55 days post-re-challenge of surviving mice, and stained for the percentage of persistent 237 CAR T cells (Tn-OTS8⁺) among the population of CD3⁺ (left panel), CD4⁺ (middle panel), or CD8⁺ cells (right panel). (**B**) IFN-γ ELISPOT of splenic T cells from untreated or CAR treated mice co-cultured with tumor cells. Spleens were removed from 237 CAR T-treated ID8*Cosmc*-KO bearing mice up to 5 months after CAR treatment (226 days for the data shown). Spleen cells were co-cultured with various C57BL/6 syngeneic cell lines including ID8 WT, ID8*Cosmc*-KO, AG104A (Tn-OTS8+), GL261, B16F10, and LLC1 tumor cells in a 96-well plate at a tumor:effector ratio of 0.3. ELISPOT assays were performed to measure IFN-γ spots 24 hours after co-culture experiment. Splenocytes from 5E5 CAR-T treated mouse or 237 CAR-T treated ID8 WT-challenged mouse that reached endpoint were used as controls, while CD3/CD28 Dynabeads were added to T cells as a positive control for T cell activation. Plates were scanned with automated ImmunoSpot analyzer. Results representative of five total experiments (with ID8Cosmc-KO and ID8-WT). Data were plotted using GraphPad Prism v9.4.1, each dot represents one mouse. Error bars are SEM.

Supplemental Figure S10



Supplemental Figure S10. Tn-antigen expression levels on tumors from CAR-treated, long-term, relapsed mice. (**A**) Ascites collected from ID8*Cosmc*-KO bearing mice that reached endpoint were stained with tetramers of biotinylated WE-scFv to examine the fraction of ascites cells that represent the antigen-positive tumor. Data were plotted according to treatment groups using GraphPad Prism v9.4.1. Each dot represents one mouse. Error bars are SEM. (**B**) Cells from ascites collected from various mice (untreated or CAR-treated) that reached endpoint criterion were grown in culture for 21 days to generate tumor cell lines. Cultured lines were stained with biotinylated WE-scFv followed by streptavidin-Alexafluor647. Histograms were overlaid against reference ID8 WT and ID8*Cosmc*-KO cell lines. (**C**) Analysis of tissue sections from 237 CAR treated mice that showed signs of relapse at >50 days post T cell transfer. Peritoneal organs were harvested from 237 CAR T treated ID8*Cosmc*-KO bearing mice that reached endpoint at Day 117 (top row, scale bar = 1 mm) and endpoint at Day 175 (bottom row, scale bar 500 μm). Immunohistochemistry was performed to visualize Tn expressing tumors, F4/80 macrophages, and CD3 T cells from FFPE tissue sections stained with biotinylated WE scFv, anti-F4/80, and anti-CD3 antibodies, respectively. H&E stained tissue sections were included as additional reference.

Supplemental Table S1

Supplemental Table S1. Pathological analyses of tissue sections from non-tumor bearing C57BL/6J mice administered with cyclophosphamide (cyc), cyc + mock T, cyc + 237 CAR T, or cyc + 5E5 CAR-T. Tissue samples were obtained from 2 different experiments, E1 and E2, at day 3 post-adoptive cell transfer.

Tissue	Microscopic Finding	Untreated		Cyclophosphamide		Cyc + mock T		Cyc + 237 CAR		Cyc + 5E5 CAR	
		E1	E2	E1	E2	E1	E2	E1	E2	E1	E2
Uterus		0	0	0	0	0	0	0	0	0	0
Ovary		NP	0	0	NP	NP	0	NP	0	0	0
Oviduct		0	0	0	0	NP	0	0	0	NP	0
Urinary bladder		0	0	0	NP	NP	NP	NP	0	0	0
Mammary gland		NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
Stomach		0	0	0	0	0	0	0	0	0	0
Small intestine		0	0	0	0	0	0	0	0	0	0
Pancreas		0	0	0	0	0	0	0	0	0	0
Adipose tissue (peritoneal)	Infiltrate, mononuclear cells	2M	2M	2M	2M	1M	2M	2M	1M	1M	1M
GALT		0	0	0	0	0	NP	0	NP	0	NP
Large intestine		0	0	0	0	0	0	0	0	0	0
Lymph node (mesenteric)		0	0	0	0	0	0	0	NP	0	NP
Spleen	Decreased cellularity, lymphocytes	0	0	1M	0	0	0	0	0	1M	NP
Kidney	Chronic progressive nephropathy	0	0	1F	0	1M	0	0	0	0	0
Adrenal gland		NP	0	0	0	0	0	NP	0	0	0
Liver	Extramedullary hematopoiesis, increased	0	1M	0	0	0	0	0	0	0	0
	Infiltrate, mononuclear cells	1M	1M	1M	1M	1M	0	1M	1M	1M	1M
Gallbladder		0	NP	0	0	0	0	0	NP	0	0
Heart		0	0	0	0	0	0	0	0	0	0
Lung	Infiltrate, mononuclear cells	0	0	0	1M	0	1M	0	0	0	0
Thymus	Decreased cellularity, lymphocytes	0	0	1D	0	NP	0	0	0	0	0
Skeletal muscle		NP	0	0	0	0	0	0	0	0	0
Brain		0	0	0	0	0	0	0	0	0	0
Carcinoma	NP=not present	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
Severity score: 0 = finding not observed; 1 = minimal; 2 = mild; 3 = moderate; 4 = marked; 5 = severe											
Distribution modifier: D = diffuse; F = focal; M = multifocal											
NP = tissue not present on slides											

Supplemental Table S2

Supplemental Table S2. Pathological analyses of tissue sections from ID8*Cosmc*-KO bearing C57BL/6J mice administered with either 237 CAR-T or 5E5 CAR-T at day 54 post-tumor inoculation. Tissue samples were harvested at day 3 post-adoptive T cell transfer.

Animal ID	Carcinoma Present? Yes/No	H&E stained tissue sections
Untreated mouse #1	Yes	Small tumor nodules adjacent to pancreas. Mitotic figures are common in the nodules along with individual and clustered small cells (lymphoid).
Untreated mouse #2	Yes	Similar to untreated mouse #1
237 CAR-treated mouse #1 at day 3 post-ACT	Yes	Small tumor nodules adjacent to pancreas with infrequent, central intratumoral necrosis. Moderate numbers of small cells (lymphocytes) infiltrating the tumor nodules. Prominent expansion of the pancreatic interlobular tissue by clear spaces (moderate edema) that is infiltrated by moderate numbers of lymphocytes and histiocytes along with a smattering of neutrophils. Patchy degeneration/necrosis (mild) of the exocrine pancreatic acinar cells. Small aggregates of mononuclear cells and/or granulocytes in the liver.
237 CAR-treated mouse #2 at day 3 post-ACT	Yes	Small tumor nodules adjacent to pancreas with rare, central intratumoral necrosis. Moderate numbers of small cells (lymphocytes) infiltrating the tumor nodules. Prominent expansion of the pancreatic interlobular tissue by clear spaces (moderate edema) that is infiltrated by small numbers of lymphocytes and histiocytes along with a smattering of neutrophils.
5E5 CAR-treated mouse #1 at day 3 post-ACT	Yes	Small tumor nodules adjacent to pancreas. Moderate numbers of small cells (lymphocytes) infiltrating the tumor nodules. Mild to moderate expansion of the pancreatic interlobular tissue by clear spaces (mild to moderate edema) that is infiltrated by moderate numbers of lymphocytes and histiocytes. Rare degeneration/necrosis (minimal) of the exocrine pancreatic acinar cells.
5E5 CAR-treated mouse #2 at day 3 post-ACT	Yes	Small tumor nodules adjacent to pancreas with infrequent, central intratumoral necrosis. Rare numbers of small cells (lymphocytes) infiltrating the tumor nodules.

Supplemental Table S3

Supplemental Table S3. Observations from ID8*Cosmc*-KO challenged C57BL/6J mice sacrificed at day 3 post-T cell transfer.

Group	Mouse #	Ascites (mL)	Weight of Pancreas with tumor nodules (mg)	Comments
Untreated	1	0.1	291.2	Bloody ascites
	2	0.1	282.2	Bloody ascites
	3	0.2	603.8	Bloody ascites
237 CAR-T	1	1.5	233.6	Milky-colored ascites; viscous pancreas surface
	2	1.6	529.4	Milky-colored ascites; viscous pancreas surface
	3	1.7	509.1	Milky-colored ascites; viscous pancreas surface
5E5 CAR-T	1	0	315.8	viscous pancreas surface
	2	0.1	390.1	viscous pancreas surface
	3	0.05	358.8	viscous pancreas surface