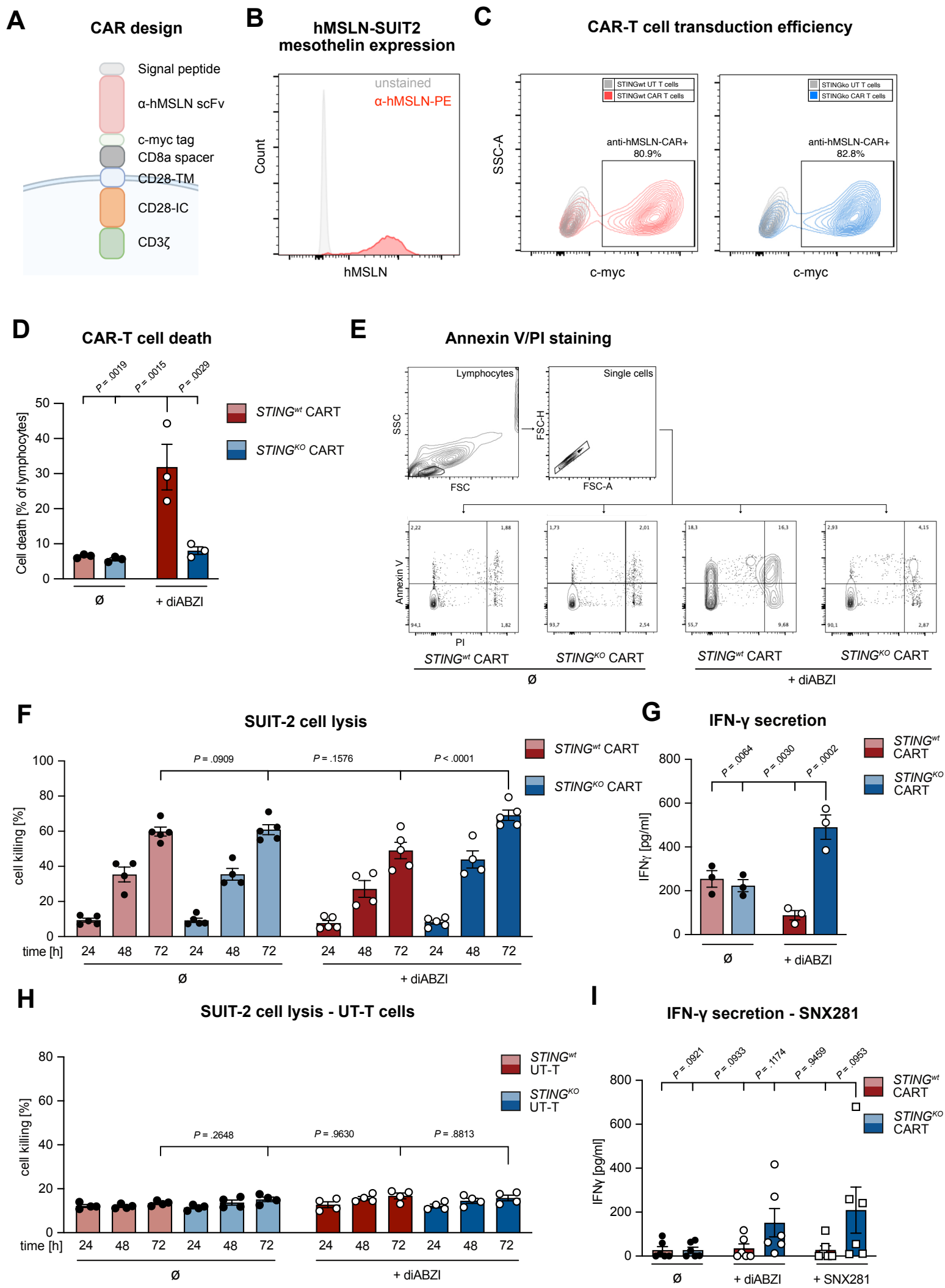
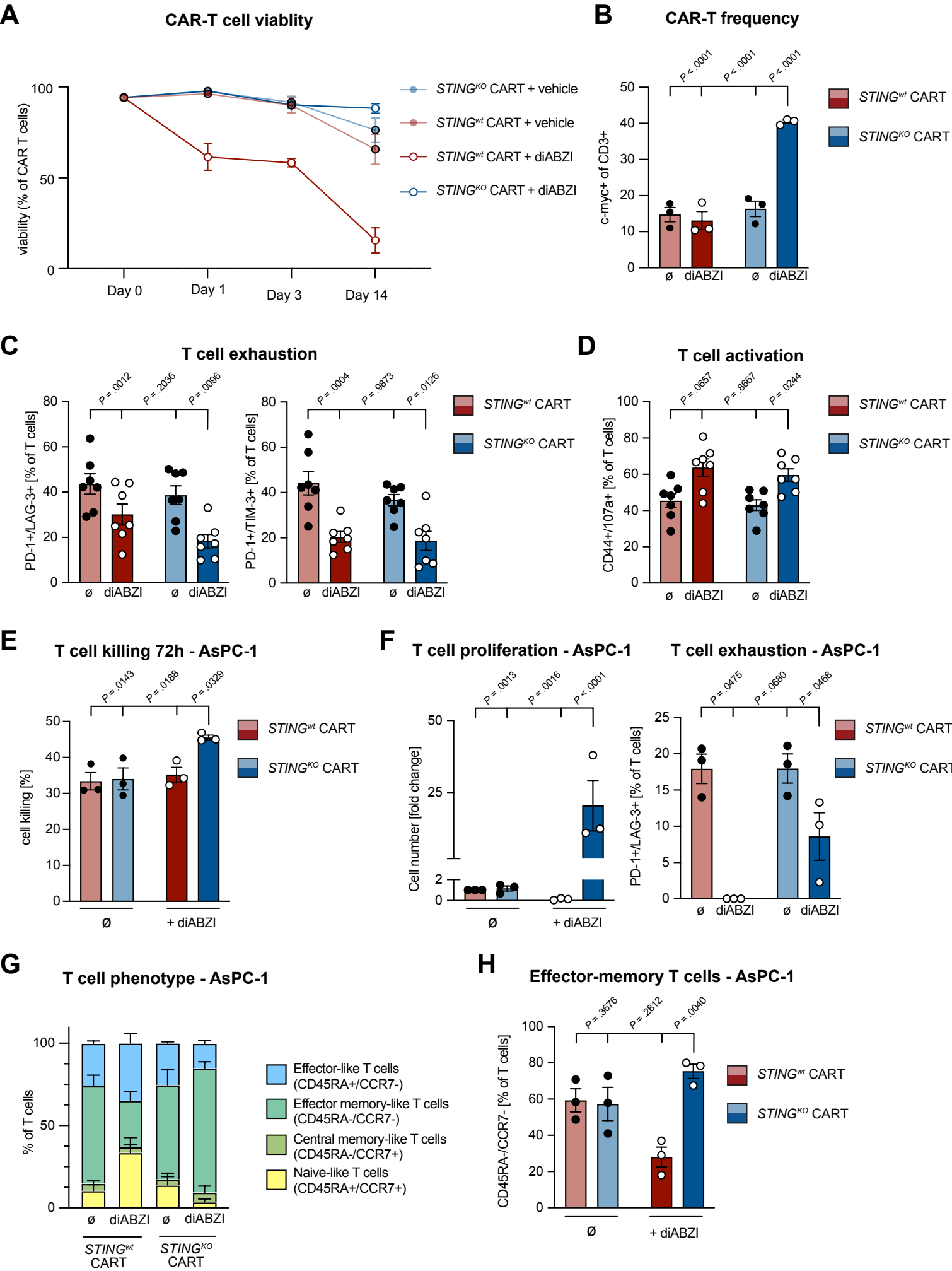


Supplementary Figure 1



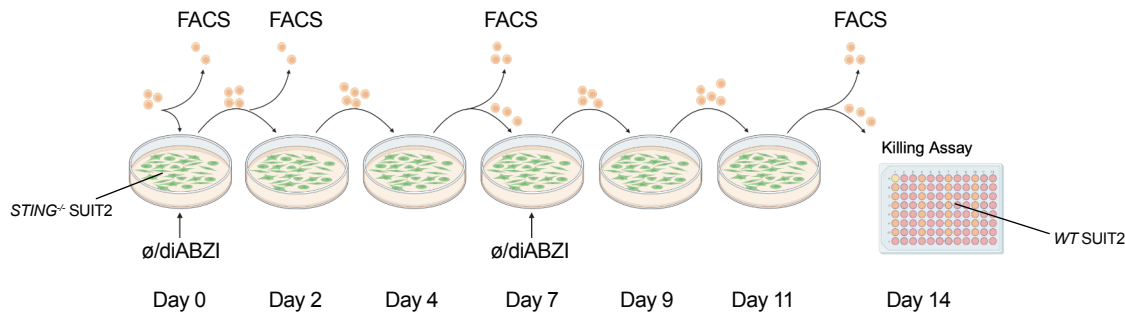
Supplementary Figure 2



Supplementary Figure 3

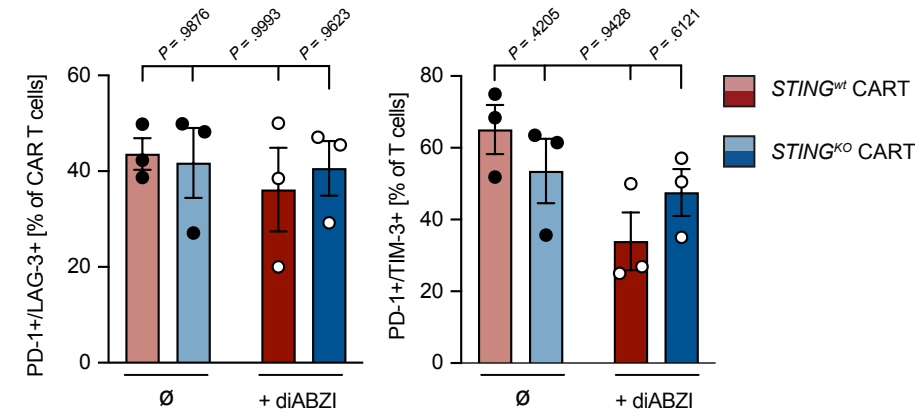
A

CAR-T cell exhaustion protocol



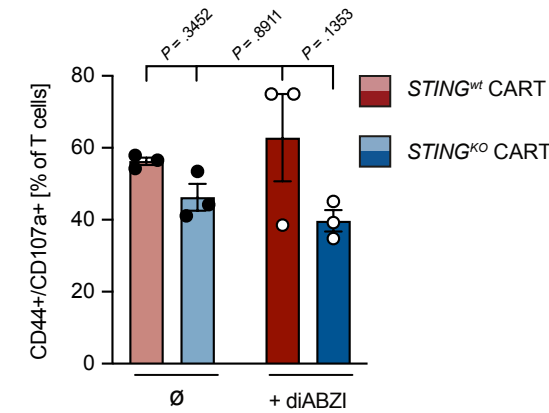
B

T cell exhaustion

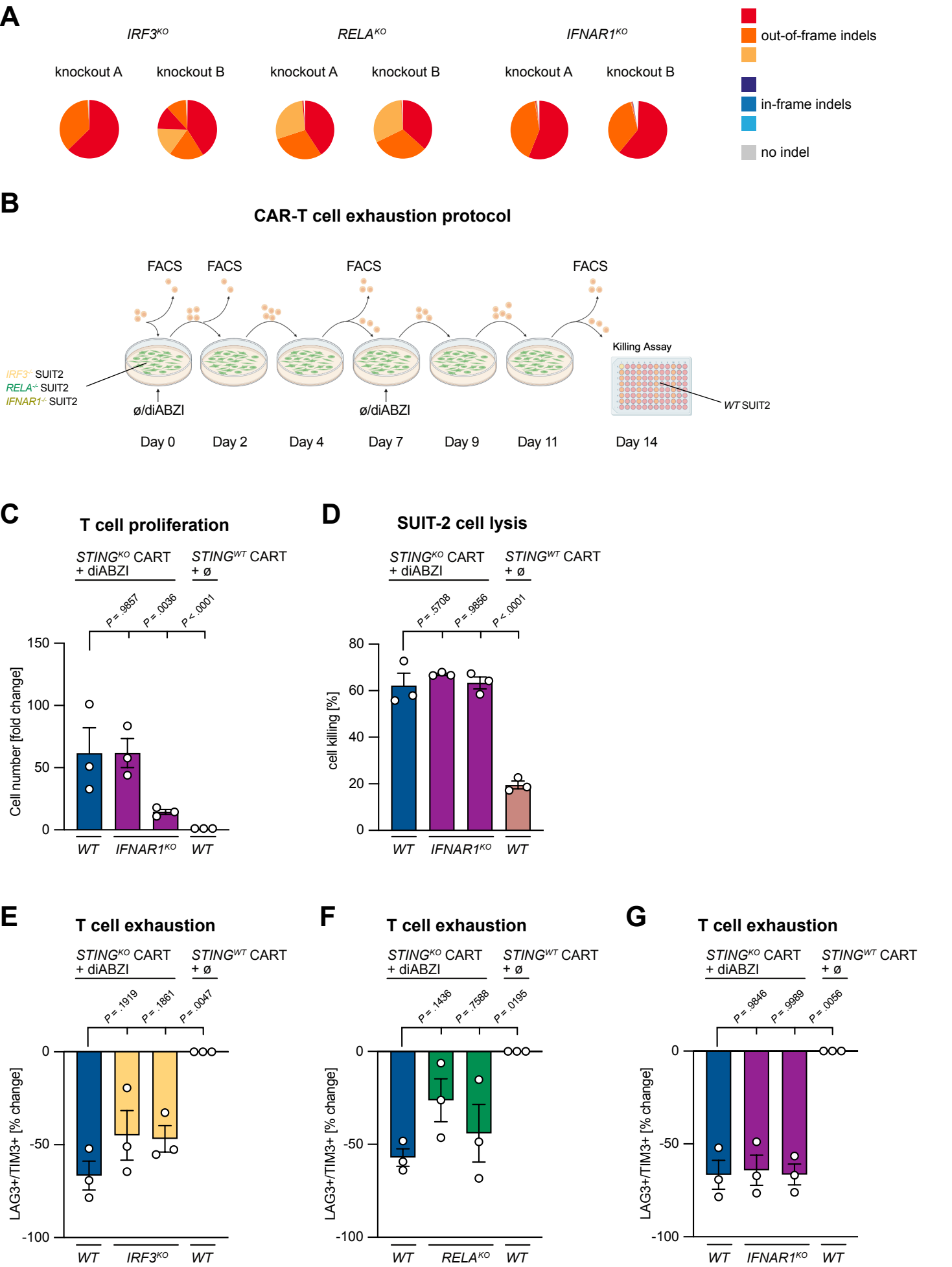


C

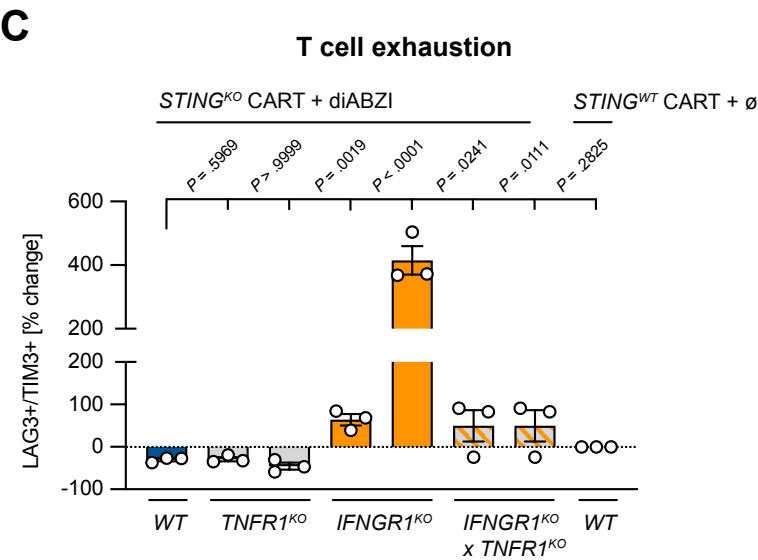
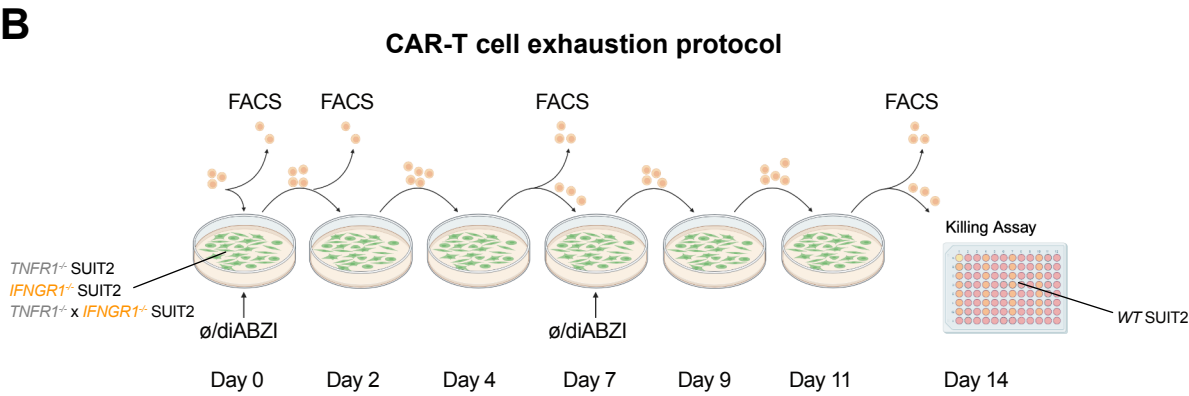
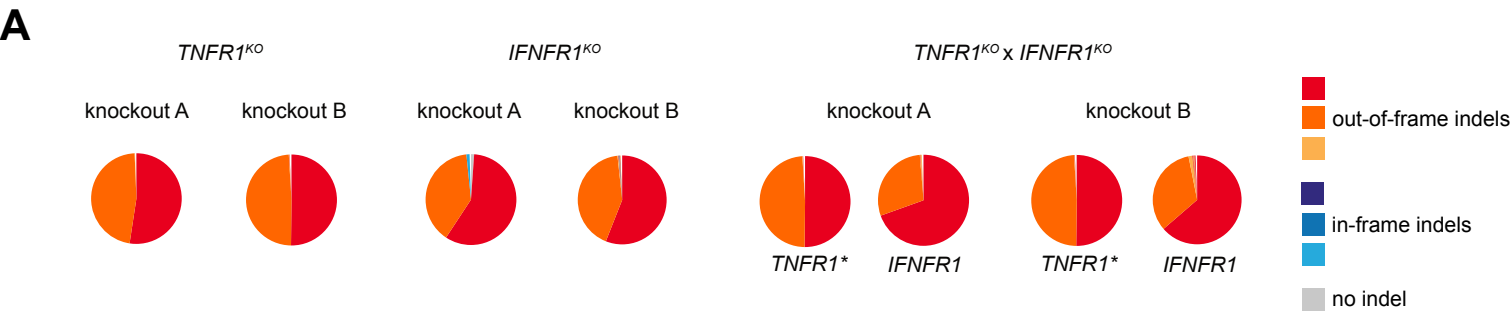
T cell activation



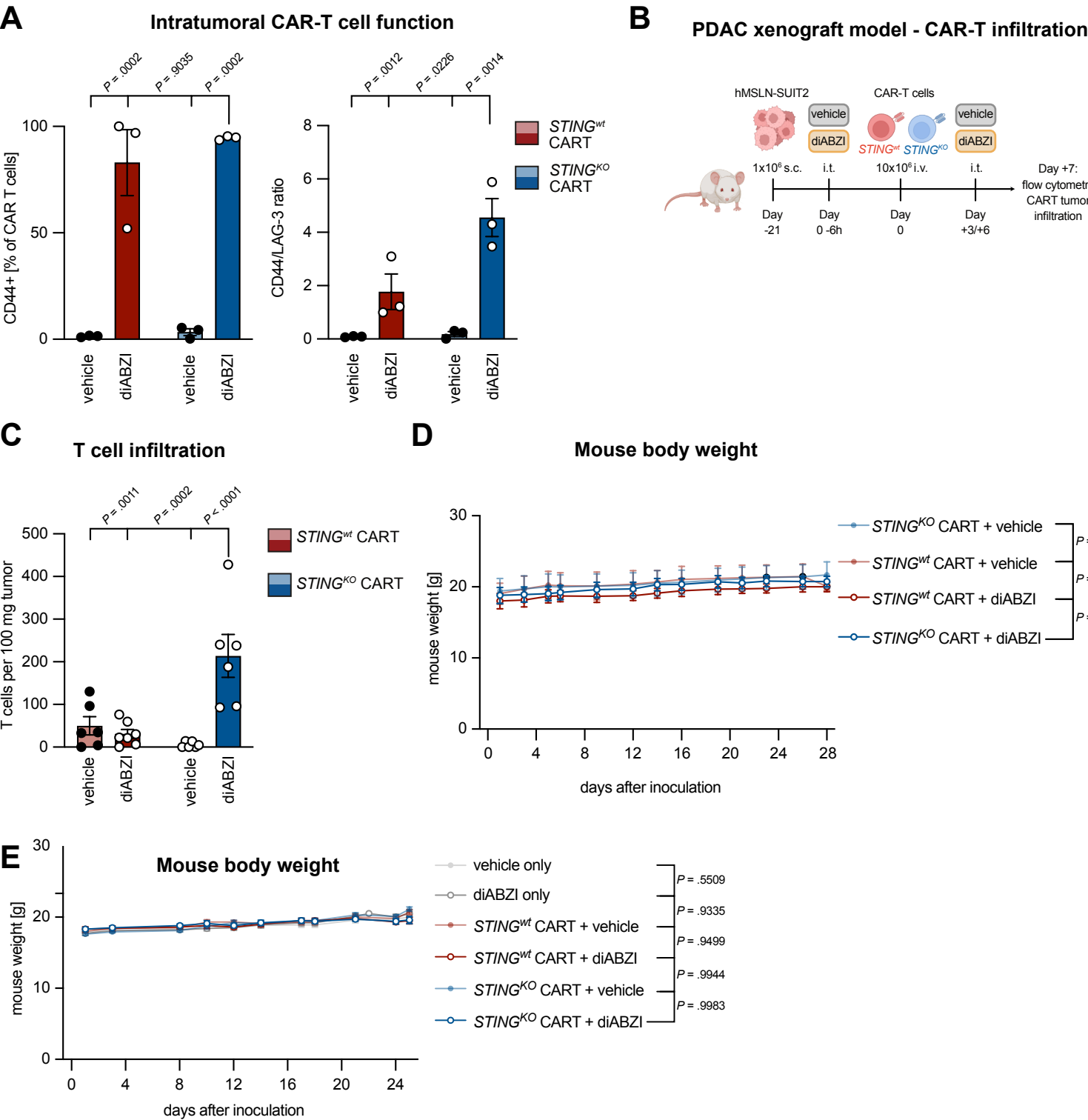
Supplementary Figure 4



Supplementary Figure 5

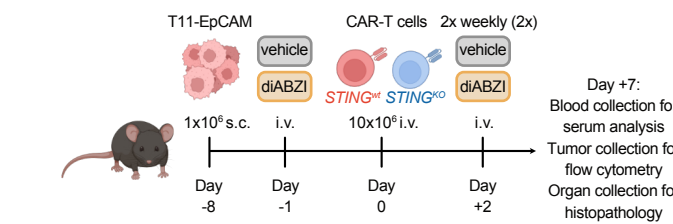


Supplementary Figure 6

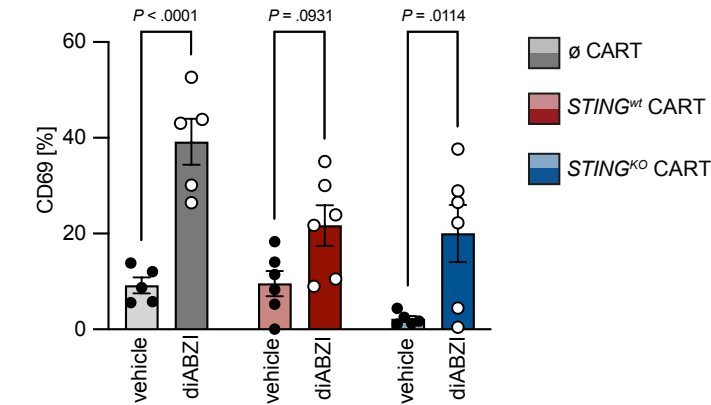


Supplementary Figure 7

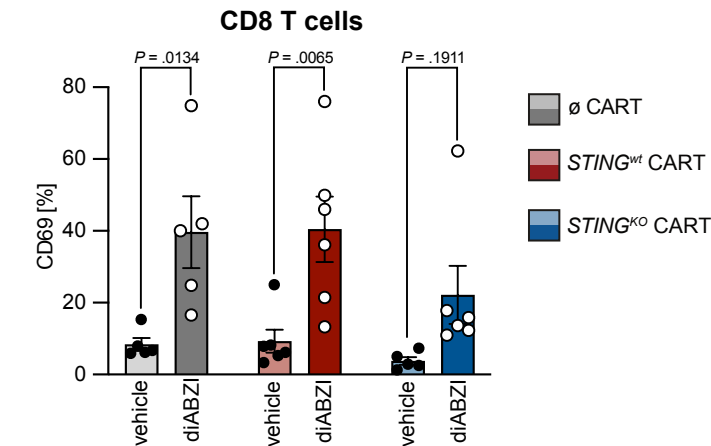
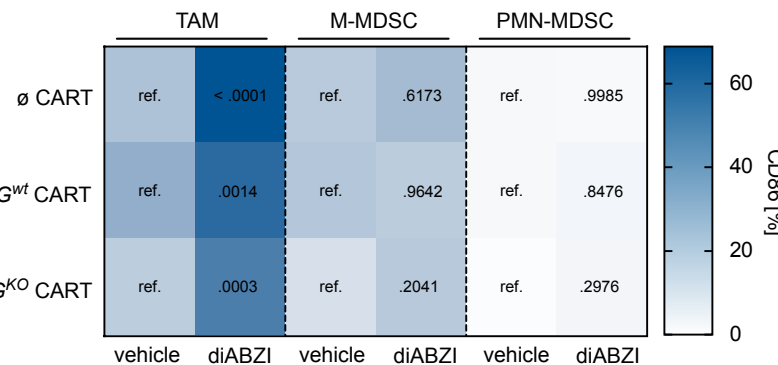
A PDAC syngeneic model - mechanism and safety



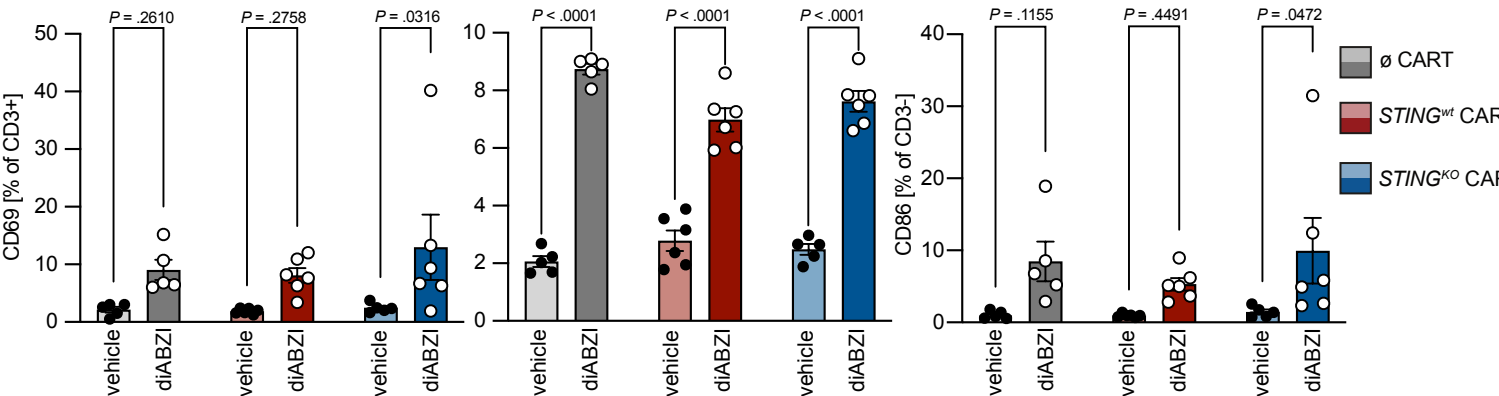
B CD4 T cells



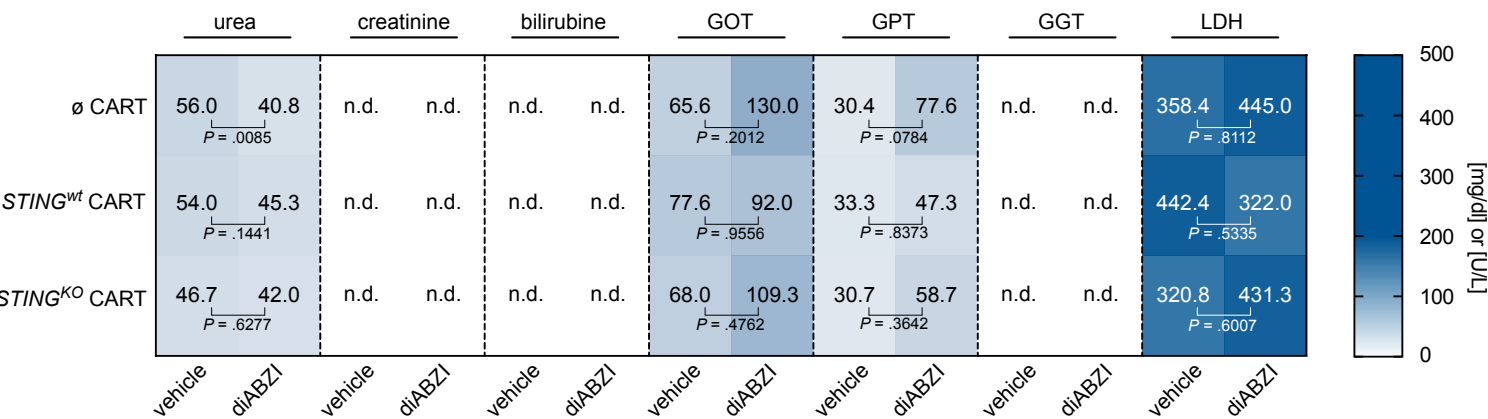
C CD86 expression intratumoral myeloid cells



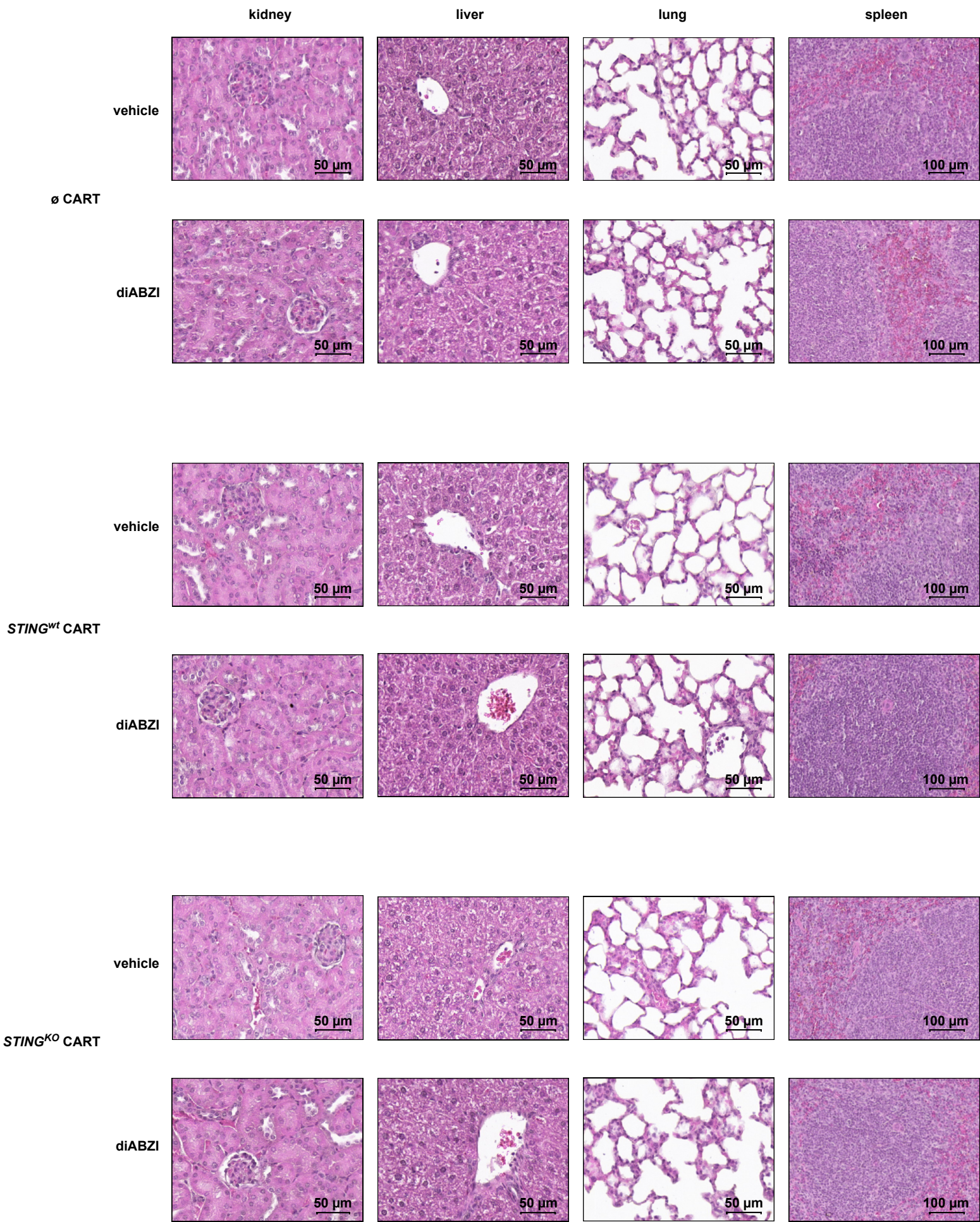
D T cell activation - dLN T cell activation - spleen



E Laboratory markers



Supplementary Figure 8



Suppl. Table 1. Serum cytokine analysis.

Cytokine	Vehicle only	diABZI only	p-value	<i>STING</i> ^{wt} CART + vehicle	<i>STING</i> ^{wt} CART + diABZI	p-value	<i>STING</i> ^{ko} CART + vehicle	<i>STING</i> ^{ko} CART + diABZI	p-value
IFN-α	0.9 (±0.9)	32.1 (±10.1)	0.007	0.0 (±0.0)	34.8 (±9.1)	0.001	0.8 (±0.8)	21.7 (±7.9)	0.077
TNF	5.9 (±2.5)	97.2 (±16.3)	<0.0001	5.0 (±1.2)	74.5 (±10.3)	<0.0001	6.8 (±0.5)	78.0 (±8.7)	<0.0001
IL-6	8.2 (±5.4)	57.9 (±11.0)	<0.001	9.9 (±6.7)	44.6 (±8.5)	0.004	2.6 (±2.6)	40.9 (±6.7)	0.002
IL-10	3.7 (±3.7)	81.9 (±43.1)	0.023	6.4 (±6.4)	32.2 (±9.8)	0.668	0.0 (±0.0)	57.8 (±20.5)	0.102
GM-CSF	16.0 (±8.3)	6.8 (±1.8)	0.498	10.5 (±4.7)	10.2 (±3.4)	>0.999	11.7 (±5.4)	2.5 (±1.5)	0.469
CXCL9	81.1 (±22.5)	4700 (±1804)	<0.001	91.2 (±8.6)	3733 (±679)	0.004	105.1 (±23.4)	3122 (±400)	0.024
CXCL10	543.3 (±213.9)	13103 (±2345)	<0.0001	415.3 (±61.2)	11586 (±1485)	<0.0001	536.8 (±94.6)	7705 (±1288)	<0.001
CCL2	97.3 (±41.4)	934.5 (±334.4)	<0.001	90.9 (±13.1)	574.8 (±78.7)	0.041	83.0 (±15.9)	650.9 (±113.4)	0.019
CCL3	3.0 (±3.0)	2.0 (±2.0)	0.981	0.0 (±0.0)	0.0 (±0.0)	>0.999	0.0 (±0.0)	6.0 (±3.7)	0.100
CCL4	7.1 (±7.1)	143.9 (±17.8)	<0.0001	0.0 (±0.0)	126.5 (±9.0)	<0.0001	0.0 (±0.0)	120.2 (±20.1)	<0.0001

Table indicates mean concentrations (pg/ml) ± SEM of indicated serum cytokines derived from *n* = 5 - 6 mice per group across treatment groups determined by flow cytometry-based multiplex assay. Statistical significance between respective vehicle- and diABZI-treated groups was determined using two-way ANOVA with Sidak's multiple comparisons test.

Supplementary Figure legends

Supplementary Figure 1: Combination of STING agonists and *STING*^{KO} CAR-T cells results in enhanced killing capacity in the context of repetitive antigen stimulation *in vitro*

(A) Graphical representation of the hMSLN-specific CAR. Abbreviations: scFv, single chain variable fragment; TM, transmembrane domain; IC, intracellular domain.

(B) hMSLN expression of transgenic SUIT-2 cells was determined by flow cytometry.

(C) Representative transduction efficiencies of *STING*^{wt} and *STING*^{KO} anti-hMSLN-CAR-T cells are shown.

(D) *STING*^{wt} and *STING*^{KO} CAR-T cells were co-incubated with SUIT-2 cells and control medium or diABZI for 24 hours, respectively. Treatment-induced cell death of CAR-T cells was assessed by Annexin V and propidium iodide staining. Data are depicted as mean ± SEM of three independent donors. Statistical analysis was conducted by two-way ANOVA with Sidak's multiple comparisons test.

(E) Representative gating strategy for experiment described in (D). Graphs show representative flow cytometric gates of one out of three independent donors.

(F and G) 3 × 10⁴ hMSLN-SUIT-2 were co-incubated with 0.6 × 10⁴ hMSLN-specific CAR-T cells of indicated genotypes with control or 0.1 μM diABZI.

(F) Cancer cell lysis was determined at the indicated time points using LDH release assay. Graph shows mean ± SEM of 4-5 independent donors. Statistical analysis was performed using two-way ANOVA with Sidak's multiple comparisons test.

(G) IFN-γ in supernatants was determined after 72h using ELISA. Data are depicted as mean ± SEM of three independent donors. Statistical analysis was conducted using two-way ANOVA with Sidak's multiple comparisons test.

(H) Repetitive antigen stimulation experiments were conducted using *STING*^{wt} and *STING*^{KO} untransduced (UT) T cells, respectively. After 14 days, cancer cell lysis was determined at the indicated time points using LDH release assay. Graph shows mean ± SEM of 4 independent donors. Statistical analysis was performed using two-way ANOVA with Sidak's multiple comparisons test.

(I) *STING*^{wt} and *STING*^{KO} CAR-T cells were treated as depicted above, but using SNX281 instead of diABZI. At day 14, 6 × 10³ CAR-T cells were co-incubated with 3 × 10⁴ hMSLN-SUIT-2 cells for 72 hours. IFN-γ in supernatants at day 3 of the final killing experiment was determined using ELISA. Data show mean ± SEM of 6 independent donors. Statistical analysis was conducted using two-way ANOVA with Sidak's multiple comparisons test.

Supplementary Figure 2: *STING*^{KO} CAR-T cells combined with diABZI show enhanced CAR-T cell functionality

(A) CAR-T cell viability was assessed at the indicated time points of CAR-T cell-cancer cell coculture using fixable viability dye staining in flow cytometry. Graph shows mean $\pm$ SEM of 3-4 independent donors.

(B) 0.2×10^6 hMSLN-CAR-T cells were coincubated with 0.2×10^6 hMSLN-SUIT-2 cells. CAR-T cell transduction efficiency was adjusted to 65-70% prior to the experiment for all treatment groups. After 24 hours, CAR expression within the T cell pool was determined by flow cytometry. Data show mean $\pm$ SEM of 3 independent donors. Statistical analysis was conducted using two-way ANOVA with Sidak's multiple comparisons test.

(C) PD-1/LAG-3 and PD-1/TIM-3 expressions were determined via flow cytometry. Graphs show mean $\pm$ SEM of 7 independent donors. Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparisons test.

(D) CD44 and CD107a expression were determined via flow cytometry. Graph shows mean $\pm$ SEM of 7 independent donors. Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparisons test.

(E-H) *STING*^{wt} and *STING*^{KO} CAR-T cells were treated as depicted above, but incubated on AsPC-1 cells. Graphs show mean $\pm$ SEM of three independent donors.

(E) At day 14, 6×10^3 CAR-T cells were co-incubated with 3×10^4 AsPC-1 cells. Cell lysis was determined using LDH release assay. Statistical analysis was conducted by two-way ANOVA with Sidak's multiple comparisons test.

(F) At day 14, absolute CAR-T cell counts were determined via flow cytometry. Fold changes were determined relative to *STING*^{wt} CAR-T cells + $\emptyset$. Statistical analysis was conducted by two-way ANOVA with Sidak's multiple comparisons test on log-transformed data. Additionally, PD-1/LAG-3 expression was determined via flow cytometry. Statistical analysis was performed using two-way ANOVA with Sidak's multiple comparisons test.

(G) CD45RA and CCR7 expression were determined via flow cytometry.

(H) Frequencies of effector-memory like T cells (CD45RA-/CCR7-) among the different CAR-T cell conditions were analyzed by flow cytometry. Statistical analysis was conducted by two-way ANOVA with Tukey's multiple comparisons test.

Supplementary Figure 3: Tumor-cell intrinsic *STING* signaling is required for enhanced functionality of *STING*^{KO} CAR-T cells

(A) Schematic illustration of T cell exhaustion protocol using repetitive antigen stimulation is demonstrated.

(B) PD-1/LAG-3 and PD-1/TIM-3 expressions were determined via flow cytometry. Graphs

show mean $\pm$ SEM of three independent donors. Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparisons test.

(C) CD44 and CD107a expression were determined via flow cytometry. Graph shows mean $\pm$ SEM of three independent donors. Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparisons test.

Supplementary Figure 4: Tumor-intrinsic IRF3 and NF- κ B support *STING*^{KO} CAR-T cell proliferation upon *STING* agonist treatment

(A) MiSeq pie charts of indicated genotypes are depicted.

(B) Schematic illustration of T cell exhaustion protocol using repetitive antigen stimulation is demonstrated.

(C-G) *STING*^{wt} and *STING*^{KO} CAR-T cells were treated as depicted above. At day 14, 6×10^3 CAR-T cells were co-incubated with 3×10^4 hMSLN-SUIT-2 cells. Graphs show mean $\pm$ SEM of three independent donors.

(C) Absolute CAR-T cell counts were determined via flow cytometry. Fold changes were determined relative to *STING*^{wt} CAR-T cells + $\emptyset$. Statistical analysis was conducted by one-way ANOVA with Dunnett's multiple comparisons test on log-transformed data.

(D) Cancer cell lysis after 72 hours determined by LDH release assay is displayed. Statistical analysis was conducted by one-way ANOVA with Dunnett's multiple comparisons test.

(E-G) LAG-3/TIM-3 co-expression was determined via flow cytometry. Percentage change of indicated conditions was calculated compared to *STING*^{wt} CAR-T cells + $\emptyset$ incubated on WT hMSLN-SUIT-2. Statistical analysis was conducted using one-way ANOVA with Dunnett's multiple comparisons test on log-transformed data of fold changes.

Supplementary Figure 5: Priming of target cell-intrinsic *STING* activation by IFN- γ and TNF is crucial for enhanced *STING*^{KO} CAR-T cell fitness

(A) MiSeq pie charts of indicated genotypes are depicted. Note that the pie chart depicting the genotype of the TNFR1 double knockout clones (indicated with an asterisk) is identical to that of single knockout clone B, as the double knockout clones were generated based on this clone.

(B) Schematic illustration of T cell exhaustion protocol using repetitive antigen stimulation is demonstrated.

(C) LAG-3/TIM-3 co-expression was determined via flow cytometry. Percentual change of indicated conditions was calculated compared to *STING*^{wt} CAR-T cells + $\emptyset$ incubated on WT hMSLN-SUIT-2. Graph shows mean $\pm$ SEM of three independent donors. Statistical analysis was conducted using one-way ANOVA with Dunnett's multiple comparisons test on log-transformed data of fold changes.

Supplementary Figure 6: Combination of *STING*^{KO} CAR-T cells and diABZI enhances antitumor efficacy, remodels the tumor microenvironment, and shows no systemic toxicity *in vivo*

(A) In the xenograft experiment, a subgroup of $n = 3$ mice per group was sacrificed at day 28, tumors were harvested and expression of CD44 and LAG-3 of intratumoral CAR-T cells was determined by flow cytometry. The frequency of CD44 among all CAR-T cells and the ratio of CD44 and LAG-3 are displayed as mean $\pm$ SEM of $n = 3$ mice per group. Statistical analysis was performed using two-way ANOVA with Sidak's multiple comparisons test.

(B) Treatment scheme for the xenograft CAR-T cell infiltration experiment.

(C) Tumors were harvested one week after CAR-T cell injection. Subsequently, absolute numbers of intratumoral CAR-T cells were determined using flow cytometry. Numbers of intratumoral CAR-T cells per 100 mg tumor are shown as mean $\pm$ SEM of $n = 6 - 7$ mice per group. Statistical significance was calculated by two-way ANOVA with Sidak's multiple comparisons test.

(D) Mouse body weight was regularly assessed during the xenograft experiment. Graph shows mean body weight of $n = 9 - 10$ mice per group. Statistical analysis was performed by two-way ANOVA with Tukey's multiple comparisons test.

(E) Mouse body weight was regularly assessed during the syngeneic subcutaneous experiment. Graph shows mean body weight of $n = 5 - 6$ mice per group. Statistical analysis was performed by two-way ANOVA with Tukey's multiple comparisons test.

Supplementary Figure 7: Combination of *STING*^{KO} CAR-T cells and diABZI enhances antitumor efficacy, remodels the tumor microenvironment, and shows no systemic toxicity *in vivo*

(A) Treatment scheme for the syngeneic subcutaneous CAR-T experiment.

(B-E) Tumors, draining lymph nodes (dLNs), spleen and blood sera were harvested one week after CAR-T cell injection.

(B) CD69 expression on intratumoral CD4 and CD8 T cells was determined by flow cytometry. Graphs show mean $\pm$ SEM of $n = 5 - 6$ mice per group. Statistical significance was calculated by two-way ANOVA with Sidak's multiple comparisons test.

(C) CD86 expression on indicated myeloid populations was determined by flow cytometry. Heatmap displays mean percentage of CD86-positive cells among indicated myeloid populations across treatment groups of $n = 5 - 6$ mice per group. Statistical significance was calculated by two-way ANOVA with Sidak's multiple comparisons test. P-values are indicated in the respective boxes.

(D) CD69 expression on pan T cells in dLNs (left) and spleen (middle) as well as CD86

expression in CD45⁺ CD3⁻ cells in dLNs were determined by flow cytometry. Graphs show mean $\pm$ SEM of $n = 5 - 6$ mice per group. Statistical significance was calculated by two-way ANOVA with Sidak's multiple comparisons test.

(E) Indicated laboratory markers were determined in mouse sera. Heatmap displays mean concentrations across treatment groups of $n = 4 - 6$ mice per group. Statistical significance was calculated by two-way ANOVA with Sidak's multiple comparisons test. Absolute values of mean concentrations and p-values are indicated in the respective boxes. n.d. = not detected.

Supplementary Figure 8: Combination of *STING*^{KO} CAR-T cells and diABZI enhances antitumor efficacy, remodels the tumor microenvironment, and shows no systemic toxicity *in vivo*

Kidneys, livers, lungs and spleens of $n = 5-6$ mice per group were harvested one week after CAR-T injection. Organ slices were stained with H&E staining. Graph shows representative image sections of organs derived from the indicated treatment groups.

Supplementary Methods

Cell lines

Human SUIT-2 pancreatic cancer cell line overexpressing human mesothelin (hMSLN-SUIT-2) as well as the virus-producing cell line 293Vec-RD114 for anti-MSLN-CAR-28 ζ production were previously described [1, 2]. Human AsPC-1 cell line expressing endogenous hMSLN were kindly provided by the Department of Medicine II, LMU University Hospital. SUIT-2 cells were cultured in DMEM medium (ThermoFisher, USA), AsPC-1 in RPMI medium (ThermoFisher, USA), both supplemented with 10 % FCS (ThermoFisher, USA), 100 IU/ml penicillin–streptomycin (ThermoFisher, USA) and 2 mM L-Glutamine (ThermoFisher, USA). Murine T110299-EpCAM cells were kindly provided by the Division of Clinical Pharmacology (LMU University Hospital) and cultured in DMEM medium with 10% FCS, 100 IU/ml penicillin–streptomycin and 2 mM L-Glutamine.

Isolation and culture of human T cells

Peripheral blood mononuclear cells (PBMCs) were isolated from leftovers from thrombocyte donation by healthy donors using Biocoll density gradient centrifugation, (Merck, Germany), as described before [3] (project number: 19-238, ethics committee of the Medical Faculty at Ludwig-Maximilians-Universität in Munich). Subsequently, PBMCs were resuspended in MACS buffer and human pan T cells were negatively selected using magnetic bead isolation following the manufacturer's instructions (Pan T cell isolation kit, human; Miltenyi, Germany). Isolated T cells were then rested overnight in human resting T cell medium consisting of RPMI 1640 (ThermoFisher, USA) supplemented with 10 % FCS (ThermoFisher, USA), 1 mM sodium pyruvate (ThermoFisher, USA), 10 mM HEPES (Sigma-Aldrich, USA), 100 IU/ml penicillin–streptomycin (ThermoFisher, USA) and 2mM L-Glutamine (ThermoFisher, USA)) as well as 5 ng/ml human recombinant IL-7 and IL-15 (both Peprotech, Germany) before performing CRISPR-Cas9 knockouts (see below). After CRISPR knockout, T cells were transferred to a 24-well plate and activated using CD3/CD28 Dynabeads (ThermoFisher, USA) in human activating T cell medium containing 50 IU/ml recombinant human IL-2 (Miltenyi, Germany), 5 ng/ml human recombinant IL-7 and IL-15 (both Peprotech, Germany) as well as 50 μ M beta-Mercaptoethanol. Cells were expanded and transferred to new wells and resupplied with fresh medium containing IL-2, IL-7 and IL-15 keeping a cell concentration of 1×10^6 cells/ml every 2 to 3 days. Approval by the responsible ethics committee and informed consent from all donors according to the declaration of Helsinki were obtained (Project number: 19-238, ethics committee of the Medical Faculty at Ludwig-Maximilians-University in Munich). Due to data safety, information on age, sex, gender, or ethnicity is not available for the study participants.

CRISPR-Cas9 knockout generation

Polyclonal CRISPR/Cas9 T cell knockouts were generated as described previously [3, 4]. Briefly, RNPs were produced by mixing synthesized, chemically stabilized tracrRNA and crRNA (both IDT, USA; 100 pmol each), annealing them at 95°C for 5 min, then letting them cool at room temperature for 30 min. tracrRNA pairs were then incubated with 40 pmol of recombinant S.p. NLS-Cas9 (IDT, USA) for at least 15 min at room temperature. For polyclonal T cell knockouts of *STING*, two crRNAs were used, so amounts of tracrRNA and Cas9 were scaled up two times. $1 - 2 \times 10^6$ cells were washed once in PBS, then resuspended in 20 μ l P3 buffer (Lonza, Switzerland), mixed with RNPs, and transferred to a 16-well nucleofection plate (Lonza, Switzerland). Cells were then electroporated using the X-unit of a 4D nucleofector (Lonza, Switzerland; program EH100). After electroporation, cells were transferred to a 24-well plate in 250 μ l RPMI 1640 medium without supplements for 30 minutes. Afterwards, 250 μ l of 2x RPMI 1640 medium with double amounts of supplements and cytokines as well as CD3/CD28 Dynabeads were added. Cells were then expanded as described above. After 10 to 14 days, T cells were analyzed for knockout efficiency by immunoblotting before being subjected to experiments.

For clonal cancer cell knockouts, RNPs were generated as described above. 2×10^4 cancer cells were subsequently resuspended in 20 μ l SG buffer (Lonza, Switzerland), mixed with RNPs and electroporated in a 16-well nucleofection plate (Lonza, Switzerland; program EH100). After 3 to 5 days, cells were subjected to minimal dilution cloning in flat-bottom 96-well plates. When colonies became visible after 2 to 3 weeks, cells were picked and subjected to deep sequencing to identify bi-allelic knockouts for the gene of interest as described before [5] or screened for complete loss of protein expression by immunoblotting. For *STING* knockout in cancer cells, cells were electroporated with RNPs consisting of two distinct crRNAs and knockout was validated using immunoblotting, as described above.

The following gRNAs were used in this study:

STING gRNA1: GCTGGGACTGCTGTAAACG; *STING* gRNA2: GACACGGGATGGATGGATGC; *IRF3* gRNA: GAGGTGACAGCCTTCTACCG; *IFNAR1* gRNA: GCGGCTGCGGACAACACCCA; *IFNGR1* gRNA: TGGTACTCCCAATATACGAT; *TNFR1* gRNA: GACCAGTCCAATAACCCCTG; *RelA* gRNA: CCTTTCCTACAAGCTCGTGG

CAR-T cell generation

The human CAR construct used was a second generation CAR vector system containing CD28 and CD3 ζ signaling domains based on the SS1 single-chain variable fragment (scFv), as previously described [1, 2]. For virus production, retroviral pMP71 vectors carrying the CAR sequence were stably introduced in the packaging cell lines 293Vec-Galv and 293Vec-RD114, as previously described [6]. Human T cell transduction has been performed as previously

published [2]. Briefly, human T cells were activated with CD3/CD28 Dynabeads in human activating T cell medium for 48h to 72h. Retroviral supernatant was then spun onto 24-well plates coated for 2h at 37°C with 10 µg/ml RetroNectin (Takara Biosciences, Japan). After centrifugation, supernatant was removed and 0.5 to 1 x 10⁶ activated T cells in 1 ml human activating T cell medium were plated on RetroNectin plates. Transduction efficiencies were determined via flow cytometry using FITC-conjugated anti-c-myc antibodies targeting a c-myc tag in the CAR at least 5 days after transduction. CAR-T cell transduction efficiencies were adjusted to 60% to 80% before initiation of experiments. Murine anti-EpCAM-mCherry CAR-T cells were generated as described previously [1]. Briefly, supernatant derived from 293Vec-Eco producer cell lines was used to transduce primary murine T cells activated with anti-CD3 (clone: 145-2C11, ThermoFisher Scientific, USA) and anti-CD28 antibodies (clone: 37.51, ThermoFisher Scientific, USA) in murine TCM supplemented with IL-2 for 24 hours. During the transduction process, murine T cells were stimulated with anti-CD3/CD28 Dynabeads and expanded in murine TCM supplemented with IL-15. Transduction efficiencies were determined via detection of mCherry-positive cells using flow cytometry and adjusted to 60% to 80% before initiation of experiments.

Animal experiments

6- to 10-week-old female C57BL/6j or NSG mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) were purchased from Janvier (France). Mice were housed on a 12-hour dark/light cycle with unlimited access to food and water. The ARRIVE guidelines for animal research (Animal Research: Reporting of In Vivo Experiments) were applied. The SUIT-2 xenograft model was established by subcutaneous injection of 1 x 10⁶ cells in PBS into the flank. For tumor growth and survival analysis, 1 x 10⁷ *STING*^{wt} or *STING*^{KO} CAR-T cells were injected intravenously at indicated time points after subdividing all mice into two groups with comparable mean tumor volumes. Subsequently, 1.5 mg/kg bodyweight of the small molecule STING agonist diABZI (Selleck Chemicals, USA) or equal volumes of vehicle control were injected intratumorally twice weekly. For infiltration analysis, diABZI was injected 14 days after tumor inoculation intratumorally. 6 hours later, 1 x 10⁷ CAR-T cells were injected intravenously. Mice were treated with two more diABZI injections within one week after T cell injection, mice were then sacrificed, and tumors were harvested for FACS analysis. The syngeneic T110299-EpCAM model was established by subcutaneous injection of 1 x 10⁶ cells in PBS into the flank. After one week, 0.75 mg/kg bodyweight of diABZI or equal volumes of vehicle control were injected intravenously, followed by intravenous injection of 1 x 10⁷ murine EpCAM-CAR-T cells derived from WT or *Sting*^{1^{-/-}} mice one day later. Afterwards, diABZI or vehicle control was intravenously administered twice weekly for three consecutive injections. For orthotopic tumor implantation, the left flank of anesthetized C57BL/6J mice was opened to gain access to the pancreas. 1 x

10⁵ T110299-EpCAM cells were injected into the pancreas. One week later, mice were intravenously injected with 1 mg/kg diABZI, followed by intravenous administration of 1x 10⁷ murine EpCAM-CAR-T cells one day later. Afterwards, diABZI or vehicle control was intravenously administered twice weekly for three consecutive injections. 14 days after CAR-T injection, mice were sacrificed and the tumors were harvested for weight measures and flow cytometric analysis. Animals were housed in specific pathogen-free facilities at the Klinikum der Universität München. All animal experiments were approved by the local regulatory agency (Regierung von Oberbayern, ROB-55.2-2532.Vet_02-20-109, ROB-55.2-2532.Vet_02-23-77). Tumor volumes were calculated as $Volume [mm^3] = (length \times width^2)/2$. Tumor measurements and endpoints were registered by an observer blinded to the treatment groups. For ethical reasons, endpoints of survival studies were defined as tumor ulceration, tumor sizes exceeding 15 mm in any dimension, weight loss above 15% or clinical signs of distress.

In vivo safety and toxicity assessment

To assess safety and toxicity *in vivo*, 1 x 10⁶ T110299-EpCAM cells were injected subcutaneously into the flank of C57BL/6J mice. After one week, 0.75 mg/kg bodyweight of diABZI or equal volumes of vehicle control were injected intravenously, followed by intravenous injection of 1 x 10⁷ murine EpCAM-CAR-T cells derived from WT or *Sting1^{-/-}* mice one day later. Afterwards, diABZI or vehicle control was intravenously administered twice weekly for two consecutive injections. One week after CAR-T administration, mouse serum was isolated and serum cytokine concentrations were analyzed using the LEGENDplex MU Cytokine Release Syndrome Panel (13-plex) kit (Biolegend, USA) according to the manufacturer's protocol. In addition, serum was analyzed by the Institute of Laboratory Medicine at the LMU University Hospital according to standard procedures. Serum samples showing signs of hemolysis were excluded from the analysis. Furthermore, spleens, lungs, kidneys and livers were harvested one week after CAR-T injections. Organs were formalin-fixed, paraffin-embedded and cut into 5 µm sections. H&E staining was performed according to standard protocols.

Antibodies and flow cytometry

Flow cytometry-based immunophenotyping of human CAR-T cells was performed by washing the cells of interest once with PBS, followed by subsequent staining for 30 min at 4°C with 1:500 Zombie Aqua Fixable Viability Dye (Invitrogen, Vienna) and 1:100-1:200 dilutions of the following staining antibodies: BV421 anti-CD3 (clone: OKT3; Biolegend, USA), BV605 anti-CD4 (clone: RPA-T4; Biolegend, USA), BV650 anti-TIM-3 (clone: F38-2E2; Biolegend, USA), BV711 anti-PD-1 (clone: EH12.2H7; Biolegend, USA), FITC anti-c-myc (clone: SH1-26E7.1.3, Miltenyi, Germany), PerCP anti-CD8 (clone: SK1; Biolegend, USA), PE anti-CD45RA (clone: HI100; Biolegend, USA), PE anti-CD107a (clone: H4A3; Biolegend, USA), PE-Cy7 anti-LAG-

3 (clone: 11C3C65; Biolegend, USA), APC anti-CCR7 (clone: G043H7; Biolegend, USA) and AF700 anti-CD44 (clone: C44Mab-5; Biolegend, USA). Cells were then washed and resuspended in PBS containing CountBright™ absolute counting beads (ThermoFisher, USA). Subsequently, cells were analyzed on a BD LSRFortessa (BD Biosciences, USA). For Annexin V/propidium iodide (PI) staining, cells were washed once in Annexin V binding buffer and then stained with 1:40 PacificBlue Annexin V (Biolegend, USA) in Annexin V binding buffer for 15 min at room temperature. Afterwards, cells were washed twice in Annexin V binding buffer and subsequently resuspended in Annexin V binding buffer containing 1:40 PI. After incubating for 5 min, cells were transferred to FACS tubes and subsequently analyzed. For flow cytometry-based immunophenotyping of lymph-nodes, spleens and tumors in the syngeneic models, cells were washed once with PBS, followed by subsequent staining for 30 min at 4°C with 1:500 Zombie Aqua Fixable Viability Dye (Invitrogen, Vienna) and 1:100-1:200 dilutions of the following staining antibodies: BV421 anti-PD-1 (clone: 29F.1A12; Biolegend, USA), BV421 anti-Ly6C (clone: HK1.4; Biolegend, USA), BV605 anti-CD45 (clone: 30-F11; Biolegend, USA), BV650 anti-F4/80 (clone: BM8; Biolegend, USA), BV711 anti-PD-L1 (clone: 10F.9G2; Biolegend, USA), FITC anti-CD3 (clone: 17A2; Biolegend, USA), PE-Cy5 anti-CD8 (clone: 53-6.7; Biolegend, USA), PE anti-CD25 (clone: PC61; Biolegend, USA), PE anti-CD11b (clone: M1/70; Biolegend, USA), PE-Cy7 anti-LAG-3 (clone: 11C3C65; Biolegend, USA), PE-Cy7 anti-Ly-6G (clone: 1A8; Biolegend, USA), APC anti-CD4 (clone: GK1.5; Biolegend, USA), AF700 anti-CD86 (clone: GL-1; Biolegend, USA) and APC-Cy7 anti-CD69 (clone: H1.2F3; Biolegend, USA). Data was analyzed using FlowJo software (BD Biosciences, USA).

Cytokine quantification

Presence of human IP-10 (=CXCL10) (BD Biosciences, USA), human interferon-(IFN-) β (R&D, USA), human IFN- γ (BD Biosciences, USA) and human IL-6 (BD Biosciences, USA) in the supernatant of co-culture assays was determined by enzyme-linked immunosorbent assay (ELISA) according to the respective manufacturer's protocols.

Immunoblotting

Cells of interest were washed once with cold PBS, and subsequently lysed in 30 to 50 μ l DISC or RIPA buffer containing protease inhibitor (Roche, Switzerland). Afterwards, samples were spun clear at 18,000 xg for 10 min at 4°C. Proteins were then denatured by adding 6x Laemmli buffer and incubating at 95°C for 10 minutes before use. Equal concentrations of total protein for each sample were separated based on molecular weight through SDS–polyacrylamide gel electrophoresis (ThermoFisher, USA) at 120 V for 75 minutes and blotted on 0.45 μ m nitrocellulose membranes (Amersham Protran, UK) at 360 mA for 60 minutes. Membranes were blocked in TBS-T supplemented with 3% BSA (Sigma-Aldrich, USA) for 30 minutes,

incubated with the primary antibodies overnight at 4°C, washed three times in TBS-T, incubated with corresponding HRP-linked secondary antibody for 2 hours at room temperature (RT), and washed again. Chemiluminescence was recorded on a Fusion Fx (Vilber, Germany) with a CCD camera directly after adding HRP substrate (Merck Millipore, USA). Contrast was enhanced in a linear fashion. Primary antibodies (anti-STING D2P2F rabbit IgG, #13647, Cell Signaling Technology, UK; anti-phospho-STING (Ser366) rabbit IgG, #19781, Cell Signaling Technology, UK and anti-beta-Actin (C4) HRP, # sc-47778 HRP, Santa Cruz Biotechnology, USA), were used at 1:1000 dilution in 3% BSA or 3% milk (for HRP-coupled antibodies).

Immunohistochemistry

Immunohistochemical staining was performed using 3 µm sections of formalin-fixed, paraffin-embedded tissue of 11 chemotherapy-naïve PDAC patients. Tissue sections were deparaffinized and rehydrated and subsequent antigen retrieval was performed by boiling in 10 mM sodium citrate (pH 6) for 10 minutes. Sections were blocked in PBS supplemented with 1% BSA-cTM (Aurion, Netherlands) and 0.1% Triton-X100 (Sigma-Aldrich, USA). STING antibody (D2P2F rabbit IgG, #13647, Cell Signaling Technology, UK) incubation was performed at 1:100 dilution in blocking buffer overnight at 4°C, followed by incubation with the secondary antibody (EnVision+ HRP. Rabbit, Agilent, USA) for 2 hours at room temperature. DAB substrate (Vector Laboratories, USA) was added, sections were counterstained with hematoxylin, dehydrated and mounted. Whole slide scans were generated with a Panoramic MIDI II slide scanner (Sysmex Deutschland GmbH, Germany). STING IHC staining was quantified using QuPath (Version: 0.5.1)¹⁸. Object classification of stroma, immune and tumor cells was trained on three different PDAC tissue samples before applied to each sample. STING-positive cells were quantified for each cell type (object class) in percent of positive cell detection. To test the toxicity of the combinatorial therapy, spleen, lung, kidney and liver were harvested from the mice, formalin-fixed and paraffin-embedded and cut into 5 µm sections. . H&E staining was performed according to standard protocols.

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