

GI CANCER

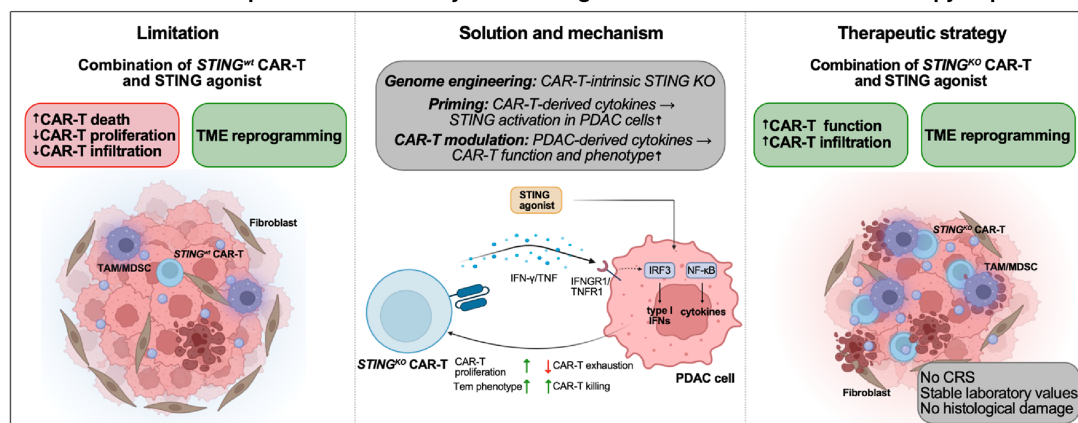
STING Ablation in T Cells Is Required for the Efficacy of STING Agonists in CAR-T Cell Immunotherapy of Pancreatic Cancer



Ignazio Piseddu,^{1,2,3} Rebekka Endres,^{1,2} Fabian Lanzl,^{1,2} Linda Hammann,^{2,3} Marleen Bérouti,¹ Matthias Thaler,^{3,4} Lisa Fahr,² Hannah Fischer,¹ Varvara Varlamova,¹ Jan Gärtig,³ Daniel Nixdorf,^{5,6} Patrick Layritz,³ Charlotte Marx,³ Christine Hörth,³ Catharina Witte,^{2,3} Alpay Bulut,^{1,2} David Illig,⁷ Anne Marie Senz,³ Lesca Holdt,⁸ Ivonne Regel,² Adrian Gottschlich,^{3,6,9} Marion Subklewe,^{5,6} Julia Mayerle,^{2,9} David Anz,^{2,3} Sebastian Kobold,^{3,9,10,11} **Andreas Linder,^{1,2,§}** and **Veit Hornung^{1,12,§}**

¹Gene Center and Department of Biochemistry, Ludwig-Maximilians-Universität München, Munich, Germany; ²Department of Medicine II, LMU University Hospital, Ludwig-Maximilians-Universität München, Munich, Germany; ³Institute of Clinical Pharmacology, LMU University Hospital, Ludwig-Maximilians-Universität München, Munich, Germany; ⁴Medizinische Klinik und Poliklinik IV, Division of Rheumatology and Clinical Immunology, LMU University Hospital, Ludwig-Maximilians-Universität München, Munich, Germany; ⁵Laboratory for Translational Cancer Immunology, LMU Gene Center, Munich, Germany; ⁶Department of Medicine III, LMU University Hospital, Ludwig-Maximilians-Universität München, Munich, Germany; ⁷Department of Pediatrics, Dr. von Hauner Children's Hospital, LMU University Hospital, Ludwig-Maximilians-Universität München, Munich, Germany; ⁸Institute of Laboratory Medicine, LMU University Hospital, Ludwig-Maximilians-Universität München, Munich, Germany; ⁹German Cancer Consortium (DKTK), Partner Site Munich, a partnership between the DKFZ Heidelberg and the University Hospital of the LMU, Munich, Germany; ¹⁰Einheit für Klinische Pharmakologie (EKLiP), Helmholtz Zentrum München - German Research Center for Environmental Health, Neuherberg, Germany; ¹¹German Center for Lung Research (DZL), Partner Site Munich, Munich, Germany; and ¹²Cluster for Nucleic Acid Sciences and Technologies – NUCLEATE, Munich, Germany

STING ablation in T cells is required for the efficacy of STING agonists in CAR-T cell immunotherapy of pancreatic cancer.



Gastroenterology

BACKGROUND & AIMS: Chimeric antigen receptor (CAR) T cells have shown great potential in hematological cancers, but lack efficacy in solid tumors, highlighting the need for novel strategies. Stimulator of interferon genes (STING) activation was shown to inflame the tumor microenvironment, but combination of STING agonists and CAR-T cells might be limited by detrimental outcomes of T cell-intrinsic STING activation. In this study, we evaluated the potential of combining STING agonists and CAR-T cells in the context of pancreatic cancer. **METHODS:** We assessed the synergy of CRISPR-Cas9-edited CAR-T cells and the STING agonist diABZI within a T cell exhaustion model in vitro and both xenograft

and syngeneic mouse models in vivo. **RESULTS:** Combination of STING-ablated CAR-T cells and diABZI resulted in enhanced cancer cell killing, increased CAR-T cell proliferation, reduced exhaustion, and expansion of an effector-memory phenotype in vitro. Mechanistically, superior CAR-T cell functionality required genetic ablation of *STING* in CAR-T cells and was dependent on cancer cell-intrinsic STING signaling on STING-agonistic treatment. Moreover, we identified a synergistic feedback loop comprising the T cell-secreted cytokines interferon-γ and tumor necrosis factor, which prime STING signaling within cancer cells, thereby potentiating the outcomes of cancer cell-intrinsic STING activation in inducing

ameliorated CAR-T cell states. Ultimately, we could demonstrate that combination of *STING* deficient CAR-T cells and diA-BZI was able to provide enhanced tumor control in both xenograft and syngeneic mouse models. This was accompanied by increased intratumoral CAR-T cell numbers and reprogramming of the tumor microenvironment in vivo. **CONCLUSIONS:** Our findings suggest that *STING* deficient CAR-T cells stand to benefit from STING agonists to improve CAR-T cell therapy for immune-deprived cancers such as pancreatic cancer.

Keywords: CAR-T Cells; STING; CRISPR-Cas9; PDAC.

Pancreatic ductal adenocarcinoma (PDAC) represents one of the most lethal cancers, with a 5-year survival rate of around 12%.¹ Despite significant advancements in targeted therapies and immunotherapies for other types of solid cancers, PDAC continues to exhibit a poor response to both conventional as well as immunotherapeutic approaches.² This resistance is largely attributed to its aggressive nature and “immune-cold” tumor microenvironment (TME), characterized by low T cell infiltration and dominant immunosuppression.² Thus, novel treatment strategies are urgently needed to improve therapeutic outcomes and patient survival.

Chimeric antigen receptor (CAR) T cells represent a novel immunotherapeutic approach that has revolutionized the treatment of hematological malignancies³; however, this remarkable success has yet to be realized in the context of solid tumors, including PDAC.⁴ The main limitations of CAR-T cell therapy in solid cancer entities comprise limited infiltration, low persistence, and intratumoral T cell dysfunction.^{3,4} Therefore, strategies to overcome these barriers are required to extend CAR-T cell efficacy to non-hematological cancers.

Targeting tumor-intrinsic pathways and the TME are possible strategies to overcome these limitations and are under active preclinical and clinical investigation.⁵ In this context, the cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) pathway, a cytosolic DNA sensor central to innate immunity, has emerged as a key driver of antitumor immune responses.^{6,7} Activation of this pathway has been described to promote cancer cell death, antigen release, dendritic cell-mediated antigen presentation and T cell priming, as well as to facilitate T cell trafficking into tumors, thereby potentiating anticancer immunity.⁷ In PDAC, treatment with STING agonists has been shown to effectively alter tumor architecture and immune profile, thereby reversing PDAC immune privilege and prolonging survival in preclinical models.^{8–10} Therefore, leveraging intratumoral STING activation in PDAC might represent a promising strategy to overcome major limitations of CAR-T cell therapy in this entity.

However, although the functionality of the cGAS-STING axis has been traditionally assigned to innate immune cells and cancer cells, recent evidence has demonstrated that this pathway is preserved, but has detrimental effects in T cells. STING agonists have been shown to promote T cell senescence and apoptosis by activating T cell-intrinsic

WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

CAR-T cells have shown great potential in hematological cancers, but lack efficacy in solid tumors, including pancreatic cancer.

NEW FINDINGS

In this study, we demonstrate that combination of CRISPR-Cas9-edited, STING-ablated CAR-T cells and synthetic STING agonists results in enhanced CAR-T cell functionality in vitro and increased tumor control, enhanced intratumoral CAR-T cell infiltration, and reprogramming of the tumor microenvironment in vivo.

LIMITATIONS

These investigations were performed in mice. Thus, clinical trials in humans investigating safety and efficacy of this approach in a human context are required.

CLINICAL RESEARCH RELEVANCE

Our data propose that the combination of *STING*^{KO} CAR-T cells and STING agonists represents a safe and powerful strategy to enhance CAR-T cell functionality and to overcome major limitations of adoptive T cell therapy in the context of poorly immunogenic solid tumors such as pancreatic cancer, thus holding promise to represent a major progress in the field of CAR-T cell therapy in solid cancers.

BASIC RESEARCH RELEVANCE

These data suggest that the combination of CRISPR-Cas9-edited therapeutic T cells with agonists of the cGAS-STING pathway is a promising strategy to increase intratumoral CAR-T cell infiltration, reduce CAR-T cell exhaustion, and to modify CAR-T cell proliferation and phenotype. Furthermore, this study provides mechanistic insights into a potent synergistic feedback loop, initiated by T cell-secreted cytokines, which sensitize cancer cells to therapeutic STING activation, thus potentiating beneficial effects on the therapeutic T cell product.

STING signaling, which limits their effects as antitumor regimens.^{11,12} Thus, a combination of STING agonists and therapeutic T cells might require the protection of CAR-T cells from detrimental outcomes of T cell-intrinsic STING activation. Technological advances in genetic engineering of

[§] Authors share co-senior authorship.

Abbreviations used in this paper: CAR, chimeric antigen receptor; cGAS, cyclic GMP-AMP synthase; hMSLN, human mesothelin; IFN, interferon; IFNGR1, interferon-gamma receptor 1; IL, interleukin; IP-10, interferon-gamma inducible protein 10; IRF3, interferon regulatory factor 3; IV, intravenous; KO, knockout; LDH, lactate dehydrogenase; MDSC, myeloid-derived suppressor cell; PDAC, pancreatic ductal adenocarcinoma; SC, subcutaneous; STING, stimulator of interferon genes; TAM, tumor-associated macrophage; TME, tumor microenvironment; TNFR1, tumor necrosis factor receptor 1; WT, wild type.

 Most current article

© 2026 The Author(s). Published by Elsevier Inc. on behalf of American Gastroenterological Association Institute. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>). 0016-5085

<https://doi.org/10.1053/j.gastro.2026.01.031>

primary human T cells holds the promise to engineer cells with high precision and low off-target effects also for clinical application. Thus, genetic modification of detrimental T cell-intrinsic pathways is a promising approach to enhance T cell-mediated anticancer responses and to facilitate the use of combination agents to overcome limitations of CAR-T cell therapy in solid tumors.

In this study, we sought to evaluate the efficacy of CAR-T cell therapy in combination with STING agonism in human and murine preclinical models of PDAC. In light of previous reports on the detrimental effects of STING activation in T cells, we focused on the role of T cell-intrinsic STING engagement and its impact on cellular immunotherapy.

Methods

Cell Lines

All human and murine cell lines used and their respective cell culture requirements are provided in the [Supplementary Methods](#) section.

Isolation and Culture of Human T Cells

Peripheral blood mononuclear cells were isolated, resuspended in MACS buffer, and human pan T cells were negatively selected using magnetic bead isolation. Isolated T cells were rested overnight in human resting T cell medium before performing CRISPR-Cas9 knockouts (see the next section). After CRISPR knockout, T cells were activated using CD3/CD28 Dynabeads (Thermo Fisher) in human activating T cell medium and expanded every 2 to 3 days.

CRISPR-Cas9 Knockout Generation

Polyclonal CRISPR-Cas9 T cell knockouts were generated as described previously.^{12,13} Briefly, RNPs were produced by mixing synthesized, chemically stabilized trans-activating CRISPR RNA (tracrRNA) and CRISPR RNA (crRNA) (both IDT; 100 pmol each), annealing them at 95°C for 5 minutes, then letting them cool at room temperature for 30 minutes. tracr:crRNA pairs were then incubated with 40 pmol of recombinant S.p. NLS-Cas9 (IDT) for at least 15 minutes at room temperature. A detailed description of knockout generation and guide RNA sequences are provided in the [Supplementary Methods](#) section.

CAR-T Cell Generation

The human CAR construct used was a second-generation CAR vector system containing CD28 and CD3ζ signaling domains, as previously described.^{14,15} Human T cell transduction has been performed as previously published.¹⁴ Murine anti-EpCAM-mCherry CAR-T cells were generated as described previously.¹⁵ A detailed protocol of human and murine CAR-T cell generation is provided in the [Supplementary Methods](#).

Animal Experiments

Six- to 10-week-old female C57BL/6J or NSG mice (NOD.Cg-Prkdcscid Il2rgtm1WjI/SzJ) were purchased from Janvier. The ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines were applied. The SUIT-2 xenograft model was established by subcutaneous (SC) injection of 10^6

cells into the flank, followed by intravenous (IV) injection of 10^7 CAR-T cells. For combination treatment, 1.5 mg/kg diABZI (Selleck Chemicals) or vehicle control was injected intratumorally. The syngeneic SC and orthotopic T11-EpCAM models were generated similarly, with 0.75 mg/kg diABZI or 1 mg/kg diABZI, respectively, vs vehicle IV and 10^7 EpCAM-CAR-T cells derived from wild-type (WT) or *Sting1*^{-/-} mice. For safety assessment, serum cytokine concentrations were analyzed using the LEGENDplex MU Cytokine Release Syndrome Panel (13-plex) kit (Biolegend) according to the manufacturer's protocol and kidney and liver function parameters were determined by the Institute of Laboratory Medicine (LMU University Hospital) according to standard procedures. All experiments were approved by local authorities.

In Vitro CAR-T Cell Killing Assays

A total of 3×10^4 human mesothelin (hMSLN)-SUIT-2 cells were plated 1 day before T cell cocubation in a 96-well flat-bottom plate in Dulbecco's modified Eagle's medium. The next day, medium was removed from tumor cells and CAR-T cells were added in an effector-to-target ratio of 1:5 in human T cell medium without cytokines. In addition, control medium or medium containing 0.1 μM diABZI was added to the coculture. After 24 hours, 48 hours, and 72 hours, supernatants were harvested and lactate dehydrogenase (LDH) release was analyzed using CyQuant LDH cytotoxicity assay (Invitrogen), according to the manufacturer's instructions. Relative LDH release was calculated as $LDH \text{ release } [\%] = 100 \times (\text{measurement} - \text{medium control}) / (\text{lysis control} - \text{medium control})$.

Repetitive Antigen Stimulation Assay

A total of 0.2×10^6 hMSLN-SUIT-2 cells were plated 1 day before the experiment. On day 0, tumor cells received 0.2×10^6 hMSLN-specific CAR-T cells and 0.1 μM diABZI (or 10 μM SNX281; MedChemExpress) or control medium. Every 2 days, supernatants containing CAR-T cells were transferred to freshly plated tumor cells, with 200 to 300 μL fresh RPMI added. On day 7, T cells were centrifuged, resuspended in fresh medium with diABZI or control, and replated. The transfer process continued every 2 days. On day 14, CAR-T cells were counted and analyzed via a 72-hour killing assay with adjusted cell numbers in an effector-to-target ratio of 1:5 to assess per-cell cytotoxic function without further diABZI addition and flow cytometry.

Antibodies and Flow Cytometry

Flow cytometry-based immunophenotyping was performed by washing the cells of interest once with phosphate-buffered saline, followed by subsequent staining for 30 minutes at 4°C with 1:500 Zombie Aqua Fixable Viability Dye (Invitrogen) and 1:100 to 1:200 dilutions of the respective staining antibodies. Subsequently, cells were analyzed on a BD LSRFortessa (BD Biosciences). Data were analyzed using FlowJo software (BD Biosciences).

Immunohistochemistry

Immunohistochemical staining was performed on 3-μm sections of formalin-fixed, paraffin-embedded tissue from 11 chemotherapy-naïve patients with PDAC. After deparaffinization, rehydration, and antigen retrieval in sodium citrate (pH 6), sections were blocked and incubated overnight at 4°C with

STING antibody (1:100) (D2P2F rabbit immunoglobulin G, #13647, Cell Signaling Technology). Secondary antibody incubation followed for 2 hours at room temperature before 3,3'-Diaminobenzidine (DAB) staining, hematoxylin counterstaining, and mounting. Whole-slide scans were generated using a Panoramic MIDI II scanner. STING immunohistochemical staining was quantified with QuPath (0.5.1)¹⁶ using a trained object classifier to distinguish stroma, immune, and tumor cells based on morphological criteria, with STING-positive cells quantified as a percentage of detected cells. Hematoxylin-eosin staining was performed according to standard protocols.

Data Visualization and Statistical Analysis

Data were visualized using GraphPad Prism version 10 (GraphPad Software Inc) and Affinity Designer (Serif). All data are presented as means and error bars show SEM. Statistical analyses were performed using Prism 10. Illustrations and treatment schemes were generated using biorender.com.

More details on the methods used in this article are provided in the [Supplementary Methods](#).

Results

Combination of STING Agonists and STING^{KO} CAR-T Cells Results in Enhanced Killing Capacity on Repetitive Antigen Stimulation In Vitro

To investigate the role of STING in T cells within the context of immunotherapy using STING agonists, we focused on PDAC, a solid tumor resistant to standard immunotherapy. As previously reported,¹⁷ we found STING highly expressed in primary human PDAC tissues from chemotherapy-naïve patients via immunohistochemistry (Figure 1A). STING was present in cancer cells, stroma, and immune cells, with the highest expression in cancer cells, followed by immune and stroma cells (Figure 1A, right), suggesting these populations as targets for STING agonists in PDAC. To assess the combination of STING-deficient CAR-T cells and STING agonists in an antigen-dependent setting, we used CAR-T cells targeting hMSLN and SUIT-2 cells transgenic for this antigen (Supplementary Figure 1A and B), a model resistant to nonengineered CAR-T cells in vivo.^{14,15} The non-nucleotide STING agonist diABZI was used for its superior pharmacodynamics over cyclic dinucleotide-based ligands.¹⁸ Using RNPs, we targeted *STING1* (encoding STING) in primary human T cells, completely abolishing STING protein expression (Figure 1B). CAR transduction efficiencies were similar in WT and *STING* knockout (*STING*^{KO}) T cells (Supplementary Figure 1C). Given that STING activation induces apoptosis in T cells, we examined diABZI-mediated apoptosis upon tumor cell encounter (Supplementary Figure 1D and E). diABZI treatment increased T cell death in *STING*^{wt} CAR-T cells, consistent with prior findings, whereas *STING*^{KO} CAR-T cells were protected from apoptosis (Supplementary Figure 1D).

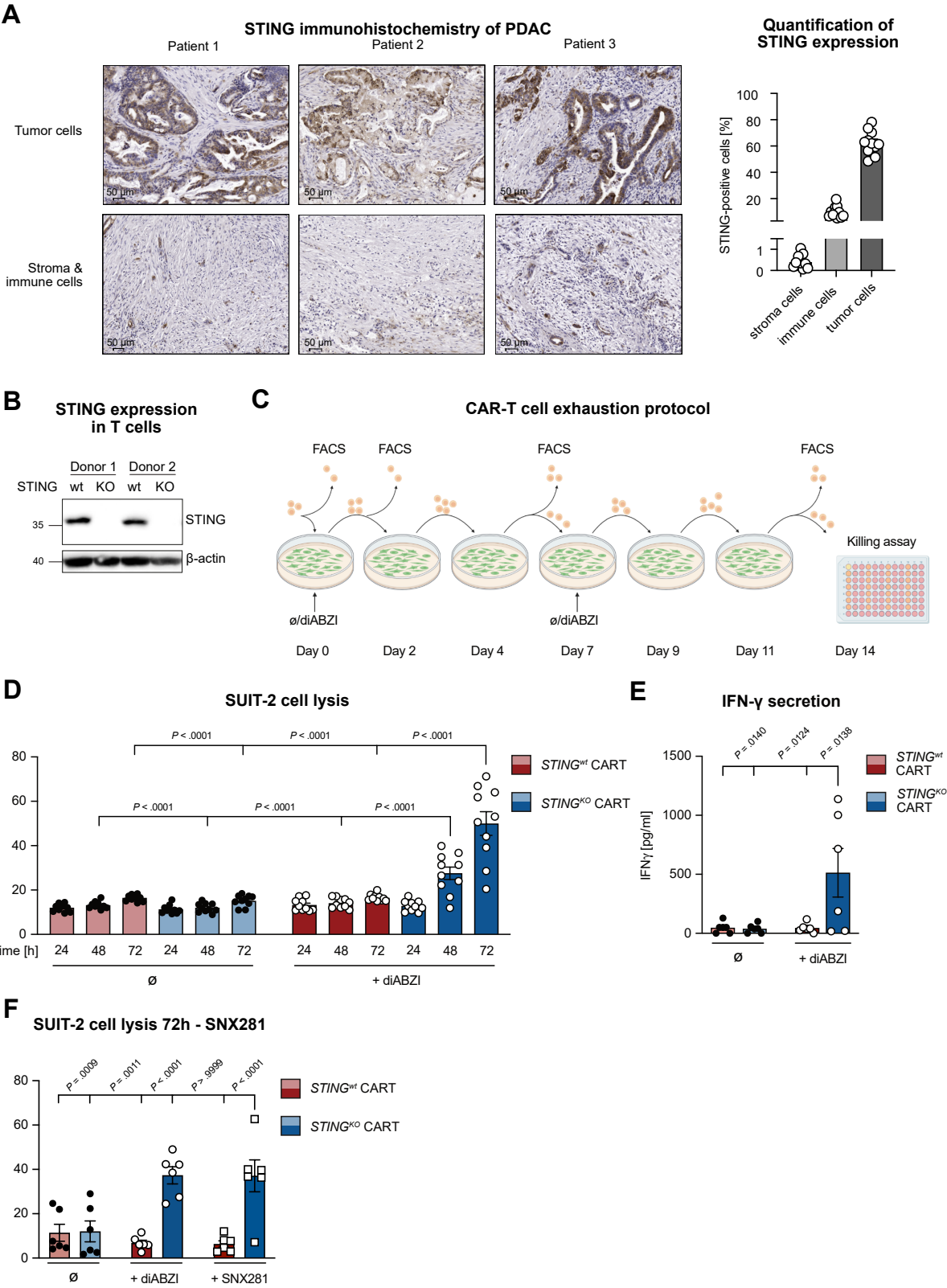
To compare cytotoxicity of *STING*^{KO} CAR-T cells and WT controls against SUIT-2 cells, we conducted killing assays

over 72 hours. Baseline killing capacity was comparable between WT and *STING*^{KO} CAR-T cells (Supplementary Figure 1F, left). In the presence of diABZI, *STING*^{KO} CAR-T cells exhibited slightly increased killing, whereas *STING*^{wt} CAR-T cells showed a moderate reduction in killing (Supplementary Figure 1F, right). Interferon (IFN)- γ secretion followed a similar pattern (Supplementary Figure 1G). To better mimic complex in vivo interactions, we used a 14-day "exhaustion" model in which CAR-T cells repeatedly encountered their cognate antigen with or without STING agonism (Figure 1C). After this period, both *STING*^{wt} and *STING*^{KO} CAR-T cells lost their killing capacity when rechallenged with fresh tumor cells in the absence of diABZI (Figure 1D, left). In contrast, *STING*^{KO} CAR-T cells retained robust cytotoxicity and IFN- γ secretion when combined with diABZI, whereas *STING*^{wt} CAR-T cells remained dysfunctional (Figure 1D, right, and Figure 1E). No benefit from diABZI was observed with untransduced T cells, confirming antigen specificity (Supplementary Figure 1H). We observed similar effects regarding cytotoxicity (Figure 1F) and IFN- γ secretion (Supplementary Figure 1I) using SNX281, an alternative small molecule STING agonist.

In summary, these results suggest that combining a STING agonist with STING-deficient CAR-T cells enhances CAR-T cell killing capacity and IFN- γ secretion under T cell exhaustion conditions in vitro.

STING^{KO} CAR-T Cells Combined With diABZI Show Enhanced CAR-T Cell Functionality

Having observed that diABZI enhanced *STING*^{KO} CAR-T cell functionality, we aimed to characterize its effects on CAR-T cells by assessing absolute numbers and phenotypes of CAR-T cells at day 14 of repetitive antigen stimulation. For comparability, CAR-T cell expansion data were normalized to *STING*^{wt} CAR-T cells without diABZI. We observed a dramatic reduction in cell numbers (Figure 2A) and viability (Supplementary Figure 2A) when *STING*^{wt} CAR-T cells were treated with diABZI, consistent with its pro-apoptotic effects. In contrast, *STING*^{KO} CAR-T cells exposed to diABZI showed a 30-fold increase in cell numbers (Figure 2A). Consistently, CAR-T frequencies dropped in all groups 24 hours after tumor cell encounter, except for *STING*^{KO} CAR-T plus diABZI, where frequencies remained substantially high (Supplementary Figure 2B), suggesting enhanced persistence and potential resistance to activation-induced cell death. Exhaustion markers TIM-3 and LAG-3 were initially low in all groups but increased over time, remaining moderate in *STING*^{KO} CAR-T cells with diABZI (Figure 2B), with similar patterns for PD-1/LAG-3 and PD-1/TIM-3 coexpression (Supplementary Figure 2C). T cell activation markers CD44/CD107a moderately increased in both diABZI-treated groups, regardless of genotype (Supplementary Figure 2D). Assessing CD45RA and CCR7 expression revealed decreased effector-like (Teff) T cells in *STING*^{KO} CAR-T cells with diABZI, whereas effector-memory-like (Tem) cells expanded compared with the other conditions (Figure 2C and D). To validate our findings in a different cell line, we used



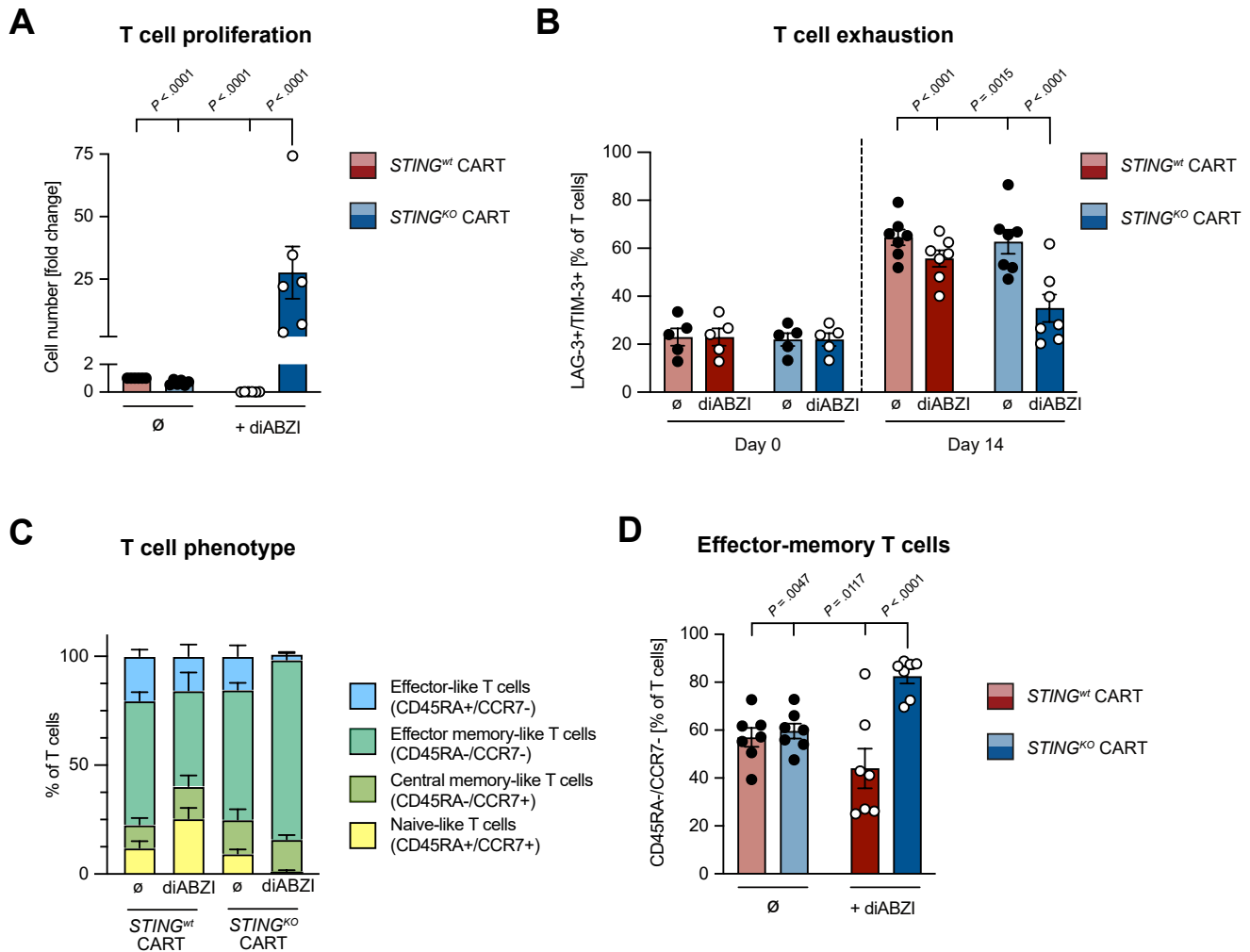


Figure 2. $STING^{KO}$ CAR-T cells combined with diABZI show enhanced CAR-T cell functionality. (A–D) At day 14 of repetitive antigen stimulation, CAR-T cells were analyzed by flow cytometry. Statistical analysis was performed using 2-way analysis of variance with Sidak’s multiple comparisons test. (A) Absolute CAR-T counts were determined. Fold changes were determined relative to $STING^{wt}$ CAR-T + $\emptyset$. Graph shows mean $\pm$ SEM of 6 independent donors. Statistical analysis was conducted on log-transformed data. (B) LAG-3/TIM-3 coexpression was determined at day 0 and day 14. Graph shows mean $\pm$ SEM of 5 (day 0) and 7 (day 14) independent donors. (C) CD45RA and CCR7 expression were determined. Graph shows mean $\pm$ SEM of 7 independent donors. (D) Frequencies of effector-memory T cells (CD45RA-/CCR7-) were analyzed. Graph shows mean $\pm$ SEM of 7 independent donors.

AsPC-1 cells, a PDAC line endogenously expressing hMSLN.¹⁹ Similar effects were observed regarding cytotoxicity (Supplementary Figure 2E), proliferation (Supplementary Figure 2F, left), exhaustion (Supplementary Figure 2F, right), and Tem phenotype formation in $STING^{KO}$

CAR-T cells plus diABZI (Supplementary Figure 2G and H), consistent with SUIT-2 results.

Taken together, $STING^{KO}$ CAR-T cells combined with diABZI exhibited improved proliferation, reduced exhaustion, and expanded Tem phenotype.

Figure 1. Combination of STING agonists and $STING^{KO}$ CAR-T cells results in enhanced killing capacity upon repetitive antigen stimulation in vitro. (A) Representative immunohistochemistry of STING in 3 of 11 PDAC tissues. Data from 11 individual samples are depicted as mean values $\pm$ SEM. (B) STING protein expression in unedited and $STING^{KO}$ T cells. β -Actin was used as loading control. One representative immunoblot of 3 independent experiments is depicted. (C) Schematic illustration of T cell exhaustion protocol. (D) Cell lysis was determined using LDH release assay. Graph shows mean $\pm$ SEM of 10 independent donors. Statistical analysis was conducted by 2-way analysis of variance (ANOVA) with Tukey’s multiple comparisons test. (E) IFN- γ in supernatants at day 3 of the final killing experiment was determined using enzyme-linked immunosorbent assay. Data show mean $\pm$ SEM of 6 independent donors. Statistical analysis was conducted using 2-way ANOVA with Sidak’s multiple comparisons test. (F) T cell exhaustion protocol was performed using SNX281 instead of diABZI. Cell lysis was determined using LDH release assay. Graph shows mean $\pm$ SEM of 6 independent donors. Statistical analysis was conducted by 2-way ANOVA with Sidak’s multiple comparisons test.

Tumor Cell-Intrinsic STING Signaling Is Required for Enhanced Functionality of *STING*^{KO} CAR-T Cells

The observation that diABZI enhanced *STING*^{KO} CAR-T cell functionality suggested a requirement for tumor cell-intrinsic STING signaling. To investigate this, we generated STING-deficient SUIT-2 cells (Figure 3A) and repeated the CAR-T cell exhaustion protocol on *STING*^{KO} tumor cells, followed by a killing experiment on *STING*^{wt} cancer cells (Supplementary Figure 3A). In this setting, *STING*^{KO} CAR-T cells combined with diABZI were as dysfunctional as other conditions in cancer cell killing and IFN- γ secretion (Figure 3B and C). CAR-T cell numbers remained unchanged in *STING*^{KO} CAR-T cells with diABZI compared with untreated controls (Figure 3D). In addition, coexpression of LAG-3/TIM-3 was strongly increased in *STING*^{KO} CAR-T cells with diABZI, similar to other conditions (Figure 3E). This pattern was also observed for PD-1/LAG-3 (Supplementary Figure 3B, left) and PD-1/TIM-3 (Supplementary Figure 3B, right). Coexpression of activation markers CD44/CD107a was comparable across all groups, with no evidence of enhanced activation in diABZI-treated groups (Supplementary Figure 3C). Effector-memory phenotype formation was unaltered in *STING*^{KO} CAR-T cells with diABZI relative to other conditions (Figure 3F).

Altogether, these findings indicate that intact tumor cell-intrinsic STING signaling is required to mediate superior CAR-T cell functionality in the combination of *STING*^{KO} CAR-T cells and diABZI.

Tumor-Intrinsic IRF3 and NF- κ B Support *STING*^{KO} CAR-T Cell Proliferation Upon STING Agonist Treatment

Because tumor cell-intrinsic STING signaling was required for superior CAR-T cell functionality, we sought to identify the downstream pathways involved. On activation, STING translocates to the Golgi, activating TBK1 and interferon regulatory factor 3 (IRF3), and inducing nuclear factor (NF)- κ B signaling²⁰ (Figure 4A). To determine whether both pathways were engaged, we measured IFN- β and interferon-gamma inducible protein 10 (IP-10) as IRF3 proxies (Figure 4B) and interleukin (IL)-6 as a NF- κ B target in cocultures of *STING*^{KO} CAR-T cells with WT or *STING*^{KO} SUIT-2 cells (Figure 4C). IFN- β , IP-10, and IL-6 levels were increased in diABZI-treated cocultures with STING-expressing cancer cells, but not with *STING*^{KO} SUIT-2. To assess the contributions of IRF3 and NF- κ B to superior *STING*^{KO} CAR-T cell functionality, we generated hMSLN-SUIT-2 cells deficient for *IRF3*, *IFNAR1*, and the NF- κ B component *RELA* (Supplementary Figure 4A). Using 2 independent KO clones per genotype, we conducted the T cell exhaustion assay followed by a killing experiment with *STING*^{KO} CAR-T cells on WT SUIT-2 (Supplementary Figure 4B). Proliferation of *STING*^{KO} CAR-T cells with diABZI was reduced when cocultured with *IRF3*- or *RELA*-deficient cancer cells (Figure 4D and E) with a more

pronounced effect on *RELA* deletion. *IFNAR1* deletion had minimal impact (Supplementary Figure 4C). Next, we evaluated *STING*^{KO} CAR-T cell killing capacity in the presence of *IRF3*, *IFNAR1*, or *RELA* deletion (Figure 4F and G; Supplementary Figure 4D). *STING*^{KO} CAR-T cells incubated with WT hMSLN-SUIT-2 served as a positive control. *IRF3* (Figure 4F) and *IFNAR1* deletion (Supplementary Figure 4D) had no effect, whereas *RELA* deletion moderately reduced cytotoxicity (Figure 4G). Exhaustion marker expression remained elevated when cocultured with *IRF3*- or *RELA*-deficient, but not *IFNAR1*-deficient SUIT-2, although the differences were not statistically significant (Supplementary Figure 4E-G).

Overall, these data indicate that therapeutic STING agonism requires both IRF3 and NF- κ B activation in cancer cells to enhance *STING*^{KO} CAR-T cell proliferation.

Priming of Target Cell-Intrinsic STING Activation by IFN- γ and TNF Is Crucial for Enhanced *STING*^{KO} CAR-T Cell Fitness

Upon antigen encounter, CAR-T cells secrete effector cytokines, particularly IFN- γ and tumor necrosis factor (TNF).²¹ Because both cytokines regulate the activation threshold of the STING pathway,^{22,23} we examined their role in enhancing CAR-T cell functionality. To assess their contribution, we analyzed STING phosphorylation in hMSLN-SUIT-2 cells (Figure 5A). Prestimulation with IFN- γ , TNF, or both increased pSTING band intensity, suggesting enhanced STING phosphorylation upon diABZI stimulation. Consequently, we observed higher IP-10 secretion (Figure 5B) as well as enhanced cell death induction on diABZI stimulation in IFN- γ /TNF-pretreated cancer cells (Figure 5C). To determine whether cytokine priming was necessary for enhanced *STING*^{KO} CAR-T cell functionality with diABZI, we generated *IFNGR1*^{-/-}, *TNFR1*^{-/-}, and double-deficient hMSLN-SUIT-2 cells (Supplementary Figure 5A). Using 2 independent KO clones per genotype, we performed the T cell exhaustion assay followed by a killing experiment in WT SUIT-2 cells (Supplementary Figure 5B). As already observed, *STING*^{KO} CAR-T cells combined with diABZI on WT SUIT-2 exhibited increased cell numbers compared with *STING*^{wt} CAR-T cells without diABZI (Figure 5D). *TNFR1* deletion slightly weakened this effect, whereas *IFNGR1* deletion, alone or with *TNFR1*, resulted in near-complete loss of proliferation, indicating a crucial role for interferon-gamma receptor 1 (IFNGR1) signaling. In addition, exhaustion marker reduction seen with *STING*^{KO} CAR-T cells and diABZI was abolished on IFNGR1 or tumor necrosis factor receptor 1 (TNFR1)/IFNGR1 deletion (Supplementary Figure 5C). Next, we evaluated CAR-T cell killing capacity under *IFNGR1* and *TNFR1* deletion. *STING*^{KO} CAR-T cells incubated with unmodified hMSLN-SUIT-2 served as a positive control, whereas unmodified CAR-T cells expanded on these cells were a negative control. *TNFR1* or *IFNGR1* deletion reduced *STING*^{KO} CAR-T cell killing capacity, whereas dual receptor deletion resulted in complete dysfunctionality,

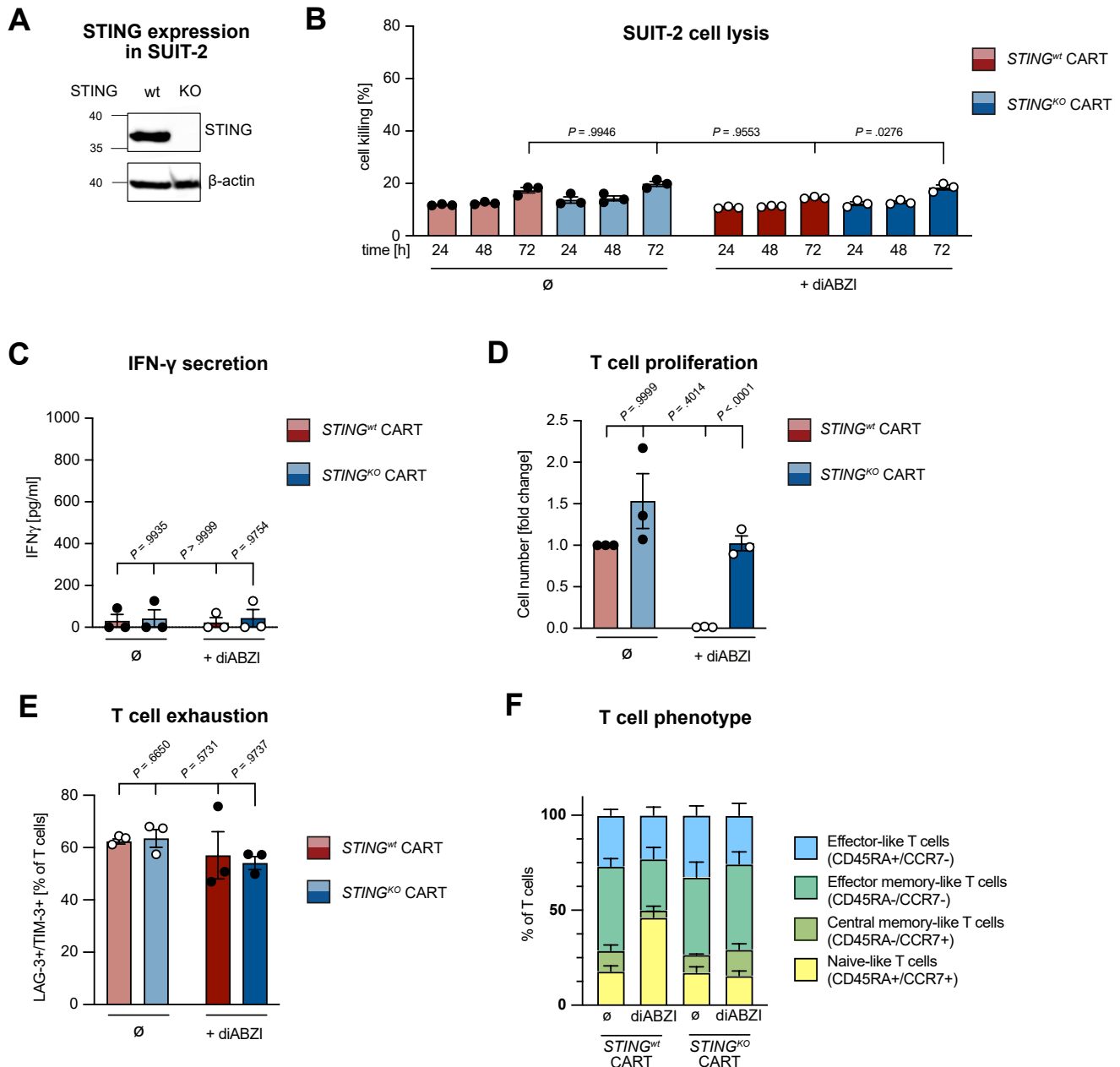


Figure 3. Tumor cell-intrinsic STING signaling is required for enhanced functionality of $STING^{KO}$ CAR-T cells. (A) Immunoblot of STING in unedited and $STING^{KO}$ hMSLN-SUIT-2 cells. β -Actin was used as loading control. One representative immunoblot of 3 independent experiments is depicted. (B–F) At day 14 of repetitive antigen stimulation, 6×10^3 CAR-T cells were co-incubated with 3×10^4 hMSLN-SUIT-2 cells. Graphs show mean $\pm$ SEM of 3 independent donors. Statistical analysis was performed using 2-way analysis of variance with Tukey's multiple comparisons test. (B) Cancer cell lysis was determined using LDH release assay. (C) IFN- γ expression in supernatants was determined using enzyme-linked immunosorbent assay. (D) Absolute CAR-T counts were determined by flow cytometry. Fold changes were determined relative to $STING^{wt}$ CAR-T cells + $\emptyset$. Statistical analysis was conducted on log-transformed data. (E) LAG-3/TIM-3 coexpression was determined using flow cytometry. (F) CD45RA and CCR7 expression were determined via flow cytometry.

similar to $STING^{wt}$ CAR-T cells without combination treatment (Figure 5E).

Taken together, these findings suggest that cancer cell-intrinsic STING signaling primed by T cell-secreted cytokines, particularly IFN- γ , is required for enhanced $STING^{KO}$ CAR-T cell fitness in combination with diABZI, highlighting the synergy between T cell-based immunotherapy and STING agonists.

Combination of $STING^{KO}$ CAR-T Cells and diABZI Enhances Antitumor Efficacy, Remodels the Tumor Microenvironment, and Shows No Systemic Toxicity In Vivo

Based on our in vitro findings, we sought to translate our results in vivo. To directly apply our approach using human T cells, we utilized immunodeficient NOD-SCID-IL2R-gamma (NSG) mice, which permit human cell

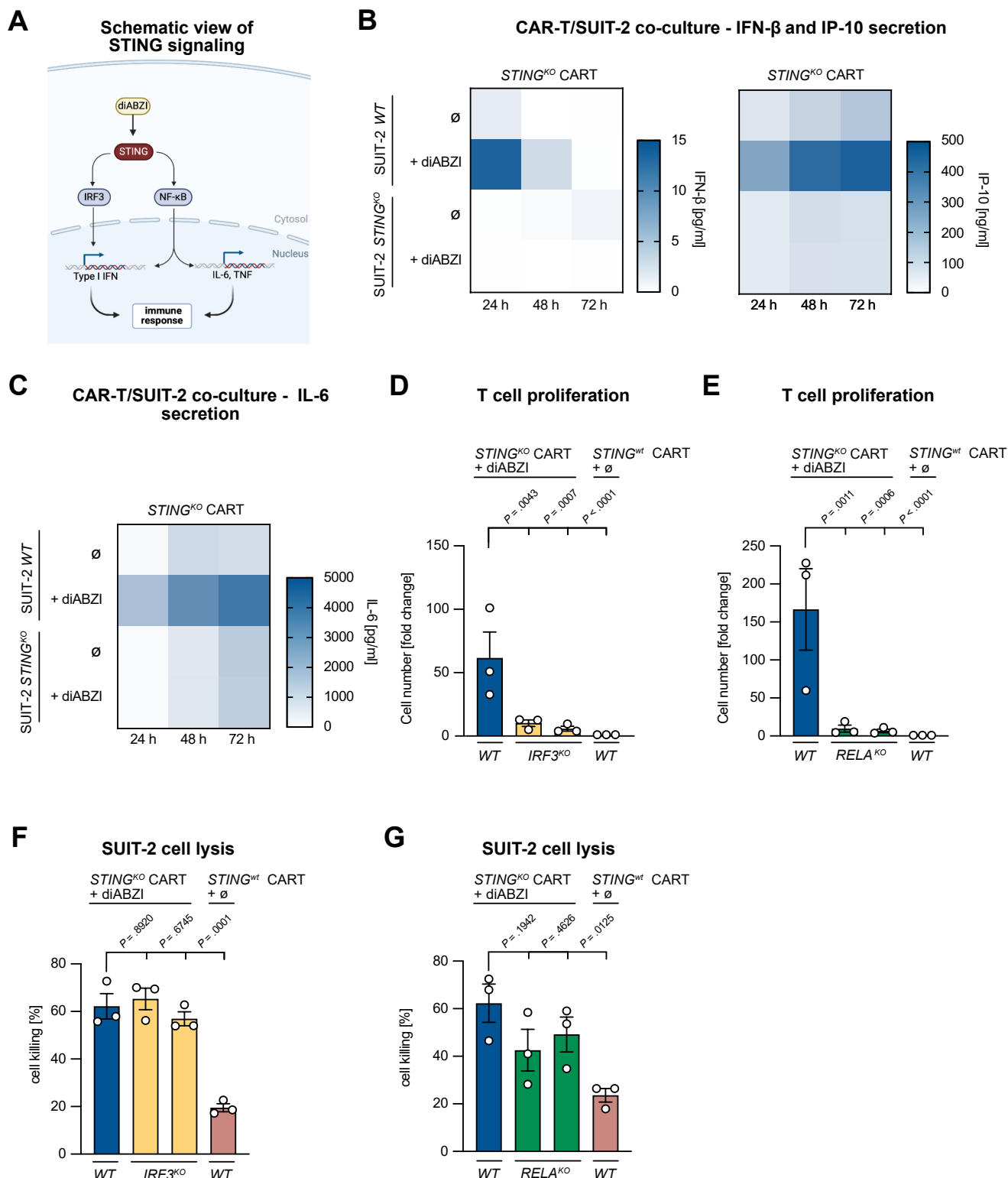


Figure 4. Tumor-intrinsic IRF3 and NF- κ B support *STING*^{KO} CAR-T cell proliferation on STING agonist treatment. (A) Scheme of STING downstream pathways. Created with biorender.com. (B and C) A total of 0.2×10^6 *STING*^{KO} CAR-T cells were co-cubated with 0.2×10^6 of either *STING*^{wt} or *STING*^{KO} hMSLN-SUIT-2 cells for 72 hours in the presence or absence of diABZI. Protein levels of IFN- β , IP-10, and IL-6 in supernatants were determined by enzyme-linked immunosorbent assay at the indicated time points. Heat maps display mean concentrations of 3 independent experiments. (D–G) At day 14 of repetitive antigen stimulation, 6×10^3 CAR-T cells were co-incubated with 3×10^4 hMSLN-SUIT-2 cells. Graphs show mean $\pm$ SEM of 3 independent donors. (D and E) 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 1-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test on log-transformed data. (F and G) Cancer cell lysis after 72 hours was determined by LDH release assay. Statistical analysis was conducted by 1-way ANOVA with Dunnett's multiple comparisons test.

engraftment. Tumor cells were implanted SC, followed by IV injection of *STING*^{wt} or *STING*^{KO} human CAR-T cells or untransduced T cells. Mice received twice-weekly intratumoral injections of diABZI or vehicle control (Figure 6A). Although *STING*^{wt} CAR-T cells and untransduced T cells with diABZI moderately reduced tumor growth, *STING*^{KO} CAR-T cells plus diABZI exhibited the strongest tumor reduction (Figure 6B). Tumors explanted on day 28 revealed increased CD44 expression in both diABZI-treated CAR-T groups (Supplementary Figure 6A, left), but the CD44-to-LAG-3 ratio was highest in the *STING*^{KO} CAR-T plus diABZI condition (Supplementary Figure 6A, right). Pre-treatment with diABZI before CAR-T injection resulted in increased intratumoral T cell numbers in this group (Supplementary Figure 6B and C). Mouse body weight remained stable across all groups (Supplementary Figure 6D).

Because xenograft models in immunodeficient mice do not capture the complex immune interactions of immunocompetent settings, and intratumoral injections have limited translational relevance, we developed a syngeneic model combining systemic (IV) diABZI administration with *STING*^{wt} or *STING*^{KO} murine CAR-T cells. We used KPC-derived T110299 PDAC cells transgenic for murine EpCAM (T11-EpCAM), and generated EpCAM-targeting murine CAR-T cells from WT or *Sting*^{1-/-} mice. Tumor cells were implanted SC, followed by IV diABZI administration one day before CAR-T cell injection (Figure 6C). Mice then received twice-weekly systemic diABZI or vehicle injections for 3 additional times. Again, systemic diABZI reduced tumor volumes only in combination with *STING*^{KO} CAR-T cells (Figure 6D). Mice maintained stable body weight (Supplementary Figure 6E).

To investigate underlying mechanisms, we performed flow cytometry-based characterization of the TME (Supplementary Figure 7A). Intratumoral CAR-T cell numbers were highest in the *STING*^{KO} CAR-T plus diABZI group (Figure 6E). In addition, endogenous intratumoral CD4⁺ and CD8⁺ T cells upregulated CD69 in diABZI-treated mice, suggesting T cell activation beyond the transferred CAR-T population (Supplementary Figure 7B). Analysis of intratumoral myeloid cells revealed a reduction in tumor-associated macrophages (TAMs) in the combination group (Figure 6F), and remaining TAMs upregulated CD86, indicative of a more proinflammatory phenotype (Supplementary Figure 7C). Within the myeloid-derived suppressor cell (MDSC) compartment, monocytic MDSCs increased, while polymorphonuclear MDSC levels remained unchanged (Figure 6F). CD86 expression was unaltered in both subsets (Supplementary Figure 7C). We further examined immune activation in secondary-lymphoid organs. In tumor-draining lymph nodes and spleen, CD69 and CD86 were upregulated on endogenous T cells and D45⁺CD3⁻ immune cells, respectively (Supplementary Figure 7D), indicating that the combination therapy not only inflames the TME but also induces activation signals in lymphoid organs. To assess safety, we analyzed serum cytokines, clinical chemistry, and histopathology. Serum levels of proinflammatory cytokines (IFN- α , TNF, IL-6)

were modestly elevated following diABZI administration but remained within tolerable limits (Figure 6G) without signs of cytokine release syndrome. CCL2, CCL4, CXCL9, and CXCL10 serum levels were also increased, while CCL3, IL-10, and granulocyte-macrophage colony-stimulating factor remained unchanged (Figure 6G, Supplementary Table 1). Liver and kidney function parameters and LDH remained stable (Supplementary Figure 7E). Histological evaluation of liver, kidney, lung, and spleen did not reveal any signs of tissue damage or inflammation (Supplementary Figure 8).

To further validate the therapeutic relevance of our findings in a more physiologic setting, we performed an orthotopic model by intrapancreatic injection of T11-EpCAM cells (Figure 6H). On termination, tumor weight was substantially reduced in the *STING*^{KO} CAR-T plus diABZI group (Figure 6I, left), and flow cytometry confirmed increased intratumoral CAR-T numbers (Figure 6I, right), indicating effective infiltration/persistence within the pancreatic TME.

Taken together, our data demonstrate that *STING*^{KO} CAR-T cells combined with diABZI enhance tumor control, increase CAR-T infiltration, and remodel the suppressive TME in immunodeficient and immunocompetent models. The combination was not associated with systemic toxicity, and its efficacy was preserved in a relevant orthotopic model that better recapitulates the anatomical features of human PDAC, supporting the translational potential of selectively uncoupling STING activation in T cells from the broader tumor and stromal compartments.

Discussion

In this study, we identified STING agonism to enhance CAR-T cell functionality in preclinical human and murine pancreatic cancer models. However, genetic ablation of *STING* in CAR-T cells was a critical prerequisite to protect them from detrimental outcomes of T cell-intrinsic STING signaling. Using a repetitive antigen stimulation model, *STING*^{KO} CAR-T cells, combined with the STING agonist diABZI, displayed superior anticancer functions. Mechanistically, cancer cell-intrinsic STING, IRF3, and NF- κ B activation, as well as IFN- γ - and TNF-mediated STING priming, formed a synergistic feedback loop: CAR-T-derived cytokines primed cancer cell-intrinsic STING signaling, enhancing its agonist-induced activation and promoting CAR-T functionality. Moreover, this approach showed potent in vivo activity across xenograft and syngeneic models in vivo. These included an orthotopic PDAC model, confirming our findings in a physiologically relevant tumor environment.

Therapeutic STING agonism represents a promising concept to enhance antitumor immune responses. In our models, combination therapy enhanced CAR-T cell infiltration/persistence, reduced TAMs while promoting a shift toward a proinflammatory phenotype, and induced activation signals in antigen-presenting cells and T cells within tumor-draining lymph nodes. These findings align with prior reports suggesting that STING agonists can remodel the TME, activate inflammatory programs in cancer-

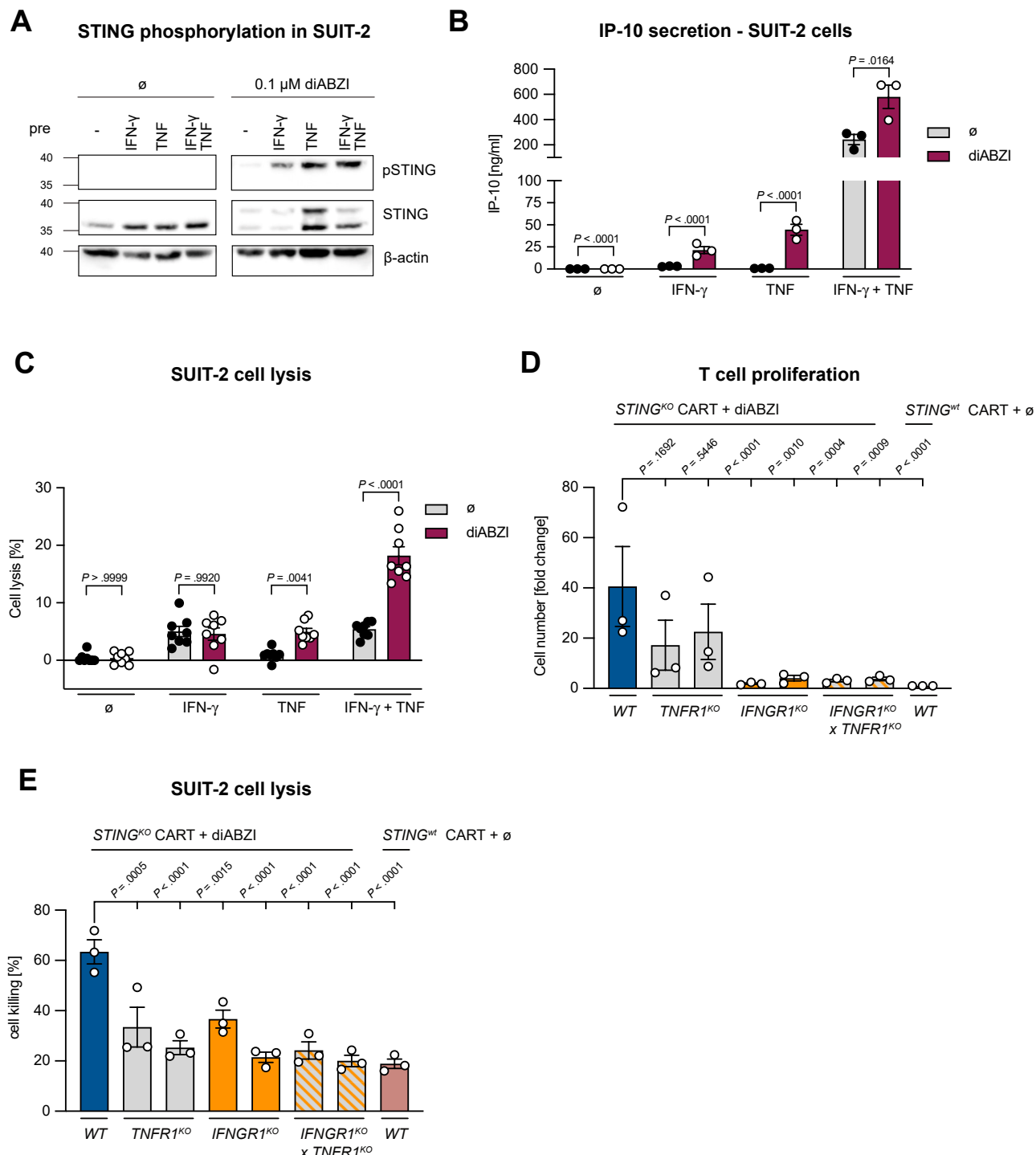
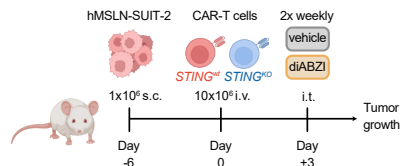
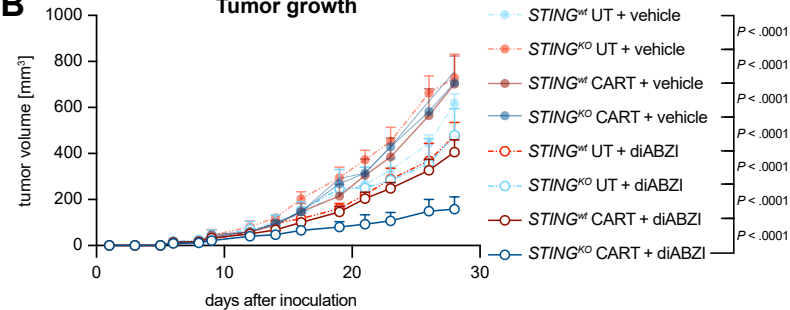


Figure 5. Priming of target cell-intrinsic STING activation by IFN- γ and TNF is crucial for enhanced *STING*^{KO} CAR-T cell fitness. (A–C) A total of 0.2×10^6 SUIT-2 cells were prestimulated for 2 hours with medium (–), 20 ng/mL IFN- γ , 20 ng/mL TNF, or the combination of both (IFN- γ /TNF). Subsequently, control medium or 0.1 μ M diABZI was added for 22 hours. (A) Expression of STING and pSTING was determined by immunoblot. β -Actin was used as loading control. One representative blot of 3 independent experiments is shown. (B) IP-10 levels in supernatants were determined by enzyme-linked immunosorbent assay. Data show mean $\pm$ SEM of 3 independent experiments. Statistical analysis was conducted by 2-way analysis of variance (ANOVA) with Sidak's multiple comparisons test on log-transformed data. (C) Cell lysis was determined using LDH release assay. Graph shows mean $\pm$ SEM of 8 independent experiments. Statistical analysis was conducted by 2-way ANOVA with Sidak's multiple comparisons test. (D and E) At day 14 of repetitive antigen stimulation, 6×10^3 CAR-T cells were co-incubated with 3×10^4 hMSLN-SUIT-2 cells. Graphs show mean $\pm$ SEM of 3 independent donors. (D) 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 1-way ANOVA with Dunnett's multiple comparisons test on log-transformed data. (E) Cancer cell lysis after 72 hours was determined by LDH release assay. Statistical analysis was conducted by one-way ANOVA with Dunnett's multiple comparisons test.

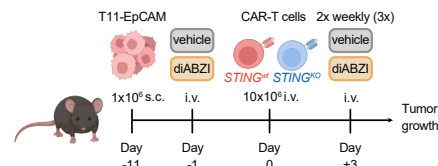
A PDAC xenograft model



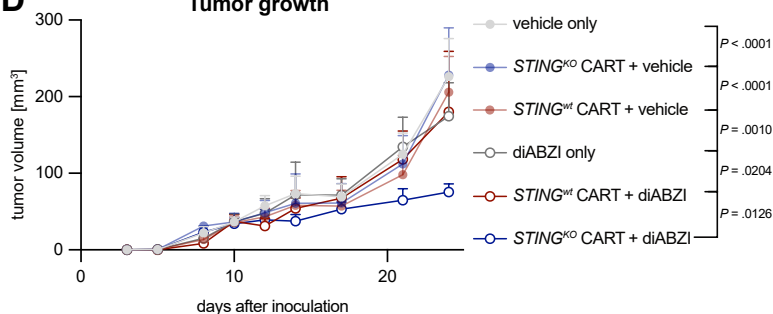
B Tumor growth



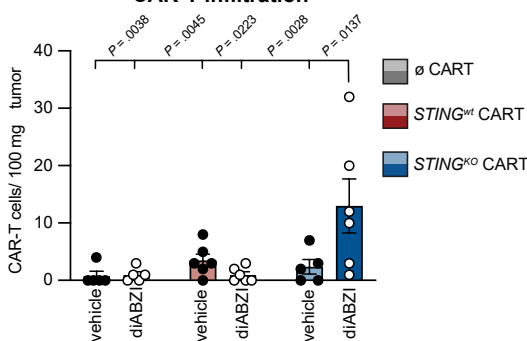
C PDAC syngeneic model



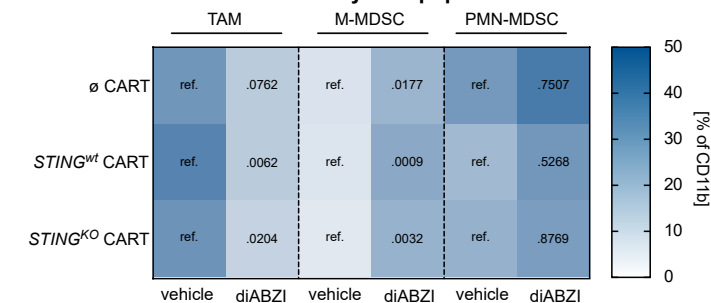
D Tumor growth



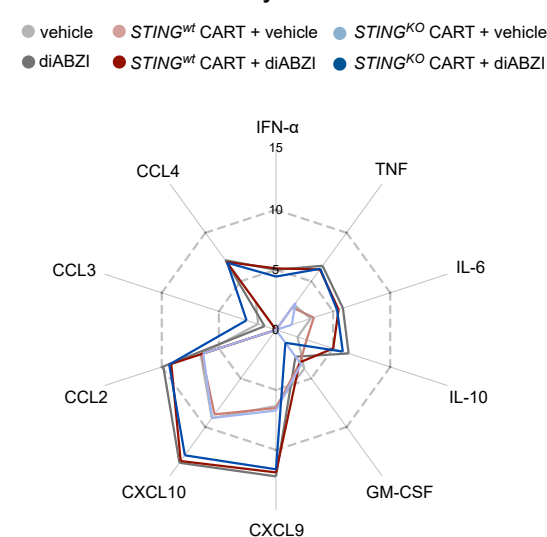
E CAR-T infiltration



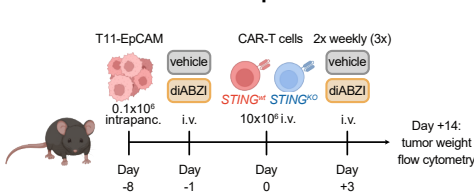
F Intratumoral myeloid populations



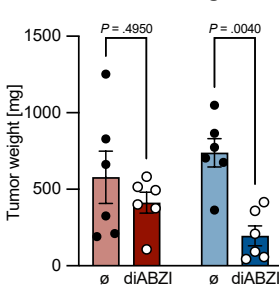
G Serum cytokines



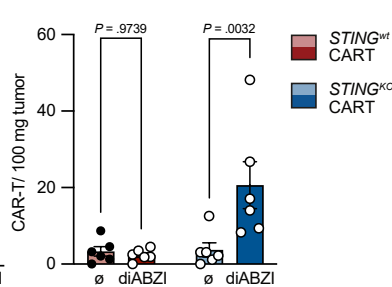
H PDAC orthotopic model



I Tumor weight



CAR-T infiltration



associated fibroblasts, and stimulate endogenous antitumor immunity, also by inducing epitope spreading, thereby counteracting frequent cancer resistance mechanisms.^{17,24,25} However, despite promising preclinical data, early clinical trials have not demonstrated sustained efficacy in solid cancers.^{24,26} Although STING agonists effectively modulate the myeloid compartment, their limited in vivo pharmacokinetics and potential detrimental effects on particularly T cells, may restrict their efficacy.²⁷ Notably, STING activation in T cells induces antiproliferative programs and apoptosis.^{12,28} To counteract this, we genetically deleted *STING* in CAR-T cells using CRISPR-Cas9, which has been established in adoptive T cell therapy to knock out inhibitory pathways in preclinical and early clinical studies without relevant off-target effects.²⁹ Thus, targeting T cell-intrinsic genes via CRISPR-Cas9 genome editing remains a safe and feasible strategy for clinical application.

A few reports have explored the combination of STING agonists and therapeutic T cells, mostly using cyclic dinucleotide agonists or intratumoral application routes,^{30–32} limiting the systemic applicability due to limited pharmacokinetics (degradation, cellular perturbation) and restricting its use in metastasized disease.^{27,33,34} Here, we used the small molecule STING agonist diABZI designed for potent STING activation with increased membrane permeability and extended in vivo half-life, even when systemically administered.¹⁸ This compound, currently under clinical investigation (NCT03843359), strongly induced T cell apoptosis unless T cells were protected by genomic *STING* knockout. *STING* deficiency was required to fully unleash the combinatorial potential of CAR-T cells and a small molecule agonist in vitro and in vivo. The local application and dosing of STING agonists in previous studies may have influenced outcomes, as therapeutic STING agonist dosing significantly impacts adaptive antitumor immunity.³⁵ Therefore, STING ablation in therapeutically administered T cells could enable stronger dosing regimens and systemic application routes.

Mechanistically, *STING*^{KO} CAR-T cells combined with diABZI showed increased proliferation, reduced exhaustion, expanded Tem populations, and decreased Teff. On diABZI treatment, cancer cells secreted IRF3- and NF- κ B-dependent cytokines, which could be amplified by priming cancer cell-intrinsic STING signaling through T cell-

derived factors. IFN- β and IL-6, proxies for IRF3 and NF- κ B activation, are both linked to T cell regulation. Type-I interferons enhance T cell proliferation and upregulate antiapoptotic programs in T cell receptor-activated T cells, while inducing apoptosis in nonactivated T cells.³⁶ IL-6 promotes T cell survival and prevents activation-induced cell death, playing a key role in T cell biology.³⁷ Our findings suggest that enhanced CAR-T functionality result from the concerted action of multiple proinflammatory cytokines induced by STING-mediated IRF3 and NF- κ B activation in cancer cells. Overall, our immunophenotypical analysis indicates that combining STING-deficient CAR-T cells with STING agonists could be a promising strategy to induce T cell-activating cytokines, leading to increased numbers of T cells with enhanced anticancer properties.^{38,39}

Regarding target antigen selection, we employed mesothelin (MSLN) in the human system—a widely studied antigen in PDAC CAR-T trials.⁴⁰ Its expression is largely restricted to healthy mesothelial tissues, but is highly upregulated in malignancies such as pancreatic, ovarian, and lung cancer.⁴¹ MSLN is considered a promising CAR target and is under clinical evaluation in multiple trials to assess safety and preliminary outcomes.⁴¹ However, MSLN-targeting CAR-T cells have shown limited persistence and efficacy in solid tumors.⁴¹ In the murine system, we used EpCAM as a target antigen based on the experiences in syngeneic models within our lab. Although biological differences between antigens, including density, shedding, spatial distribution, and susceptibility to antigen escape,⁴² may influence CAR-T efficacy, the observed improvements in tumor control were consistent despite this antigen switch, supporting the robustness of our combination strategy. Importantly, our combination substantially improved tumor control in models highly resistant to CAR treatment alone, highlighting its potential for clinical translation. In addition, our approach exhibited a favorable safety profile, with no evidence of cytokine release syndrome (CRS) compared with preclinical CRS models or extrapolated to the human setting,^{43,44} and no treatment-related organ toxicities based on clinical chemistry and histopathology. These data suggest that selective uncoupling of STING signaling in T cells, while maintaining its activity in other compartments, allows for safe and effective systemic application of STING agonists.

Figure 6. Combination of *STING*^{KO} CAR-T cells and diABZI enhances antitumor efficacy, remodels the TME, and shows no systemic toxicity in vivo. (A) Treatment scheme for xenograft CAR-T experiment. (B) Tumor growth is displayed. Graph shows mean $\pm$ SEM of pooled data from 2 independent experiments with n = 5–6 mice per CAR-T group and n = 3 mice per untransduced T group. Statistical analysis was performed using 2-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test. P values for the last measurement are depicted. (C) Treatment scheme for syngeneic subcutaneous CAR-T experiment. (D) Tumor growth is displayed. Graph shows mean $\pm$ SEM of n = 5–6 mice per group. Statistical analysis was performed using 2-way ANOVA with Dunnett's multiple comparisons test. P values for the last measurement are depicted. (E–G) Tumors and blood sera were harvested 1 week after CAR-T injection. (E) Absolute counts of intratumoral CAR-T cells were determined using flow cytometry. Numbers per 100-mg tumor are shown as mean $\pm$ SEM of n = 5–6 mice per group. Statistical significance was calculated by 2-way ANOVA with Sidak's multiple comparisons test. (F) Heatmap displays mean frequencies of indicated populations among groups of n = 5–6 mice per group. Statistical significance was calculated by 2-way ANOVA with Sidak's multiple comparisons test. P values are indicated in the respective boxes. (G) Radar plot shows log2-transformed mean serum levels of indicated cytokines of n = 5–6 mice per group. (H) Treatment scheme for syngeneic orthotopic CAR-T experiment. (I) Tumors were harvested 2 weeks after CAR-T injection. Tumor weight was determined and absolute numbers of intratumoral CAR-T cells were analyzed using flow cytometry. Data are shown as mean $\pm$ SEM of n = 6 mice per group. Statistical significance was calculated by 2-way ANOVA with Sidak's multiple comparisons test.

Limitations

First, we utilized human cell line- and KPC-derived models, which may not fully recapitulate human PDAC heterogeneity and complexity. Second, although our approach demonstrated efficacy and safety, further evaluation in more advanced models such as patient-derived xenografts or humanized mouse systems would be desired to validate translatability. Last, long-term effects on CAR-T memory and potential immunopathology were not addressed and warrant future investigation in vivo.

Conclusions

In conclusion, our data propose that STING ablation in CAR-T cells is a promising strategy to harness the immunostimulatory potential of STING agonists. This combination improves CAR-T functionality and persistence and inflames the TME while preserving safety. These findings offer a compelling rationale for clinical exploration of *STING*^{KO} CAR-T cells in combination with STING agonists for the treatment of poorly immunogenic solid tumors such as PDAC.

Declaration of Generative AI and AI-Assisted Technologies in the Writing Process

Portions of this manuscript were edited for clarity and conciseness using OpenAI's ChatGPT. The AI-assisted modifications were restricted to improving readability and conciseness, with all scientific content and conclusions remaining the sole responsibility of the authors.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at <https://doi.org/10.1053/j.gastro.2026.01.031>.

References

1. Siegel RL, Giaquinto AN, Jemal A. Cancer statistics, 2024. *CA Cancer J Clin* 2024;74:12–49.
2. Espinet E, Klein L, Puré E, et al. Mechanisms of PDAC subtype heterogeneity and therapy response. *Trends Cancer* 2022;8:1060–1071.
3. Sterner RC, Sterner RM. CAR-T cell therapy: current limitations and potential strategies. *Blood Cancer J* 2021;11:69.
4. Rafiq S, Hackett CS, Brentjens RJ. Engineering strategies to overcome the current roadblocks in CAR T cell therapy. *Nat Rev Clin Oncol* 2020;17:147–167.
5. Young RM, Engel NW, Uslu U, et al. Next-generation CAR T-cell therapies. *Cancer Discov* 2022;12:1625–1633.
6. Woo SR, Fuertes MB, Corrales L, et al. STING-dependent cytosolic DNA sensing mediates innate immune recognition of immunogenic tumors. *Immunity* 2014;41:830–842.
7. Zhu Y, An X, Zhang X, et al. STING: a master regulator in the cancer-immunity cycle. *Mol Cancer* 2019;18:152.
8. Jing W, McAllister D, Vonderhaar EP, et al. STING agonist inflames the pancreatic cancer immune micro-environment and reduces tumor burden in mouse models. *J Immunother Cancer* 2019;7:115.
9. Vonderhaar EP, Barnekow NS, McAllister D, et al. STING activated tumor-intrinsic type I interferon signaling promotes CXCR3 dependent antitumor immunity in pancreatic cancer. *Cell Mol Gastroenterol Hepatol* 2021;12:41–58.
10. Ager CR, Boda A, Rajapakshe K, et al. High potency STING agonists engage unique myeloid pathways to reverse pancreatic cancer immune privilege. *J Immunother Cancer* 2021;9:e003246.
11. Wu J, Chen Y-J, Dobbs N, et al. STING-mediated disruption of calcium homeostasis chronically activates ER stress and primes T cell death. *J Exp Med* 2019;216:867–883.
12. Kuhl N, Linder A, Philipp N, et al. STING agonism turns human T cells into interferon-producing cells but impedes their functionality. *EMBO Rep* 2023;24:e55536.
13. Linder A, Bauernfried S, Cheng Y, et al. CARD8 inflammasome activation triggers pyroptosis in human T cells. *EMBO J* 2020;39:e105071.
14. Cadilha BL, Benmebarek MR, Dorman K, et al. Combined tumor-directed recruitment and protection from immune suppression enable CAR T cell efficacy in solid tumors. *Sci Adv* 2021;7:eabi5781.
15. Lesch S, Blumenberg V, Stoiber S, et al. T cells armed with C-X-C chemokine receptor type 6 enhance adoptive cell therapy for pancreatic tumours. *Nat Biomed Eng* 2021;5:1246–1260.
16. Bankhead P, Loughrey MB, Fernández JA, et al. QuPath: open source software for digital pathology image analysis. *Sci Rep* 2017;7:16878.
17. Kabashima A, Matsuo Y, Ito S, et al. cGAS-STING signaling encourages immune cell overcoming of fibroblast barricades in pancreatic cancer. *Sci Rep* 2022;12:10466.
18. Ramanjulu JM, Pesiridis GS, Yang J, et al. Design of amidobenzimidazole STING receptor agonists with systemic activity. *Nature* 2018;564:439–443.
19. Argani P, Iacobuzio-Donahue C, Ryu B, et al. Mesothelin is overexpressed in the vast majority of ductal adenocarcinomas of the pancreas: identification of a new pancreatic cancer marker by serial analysis of gene expression (SAGE)1. *Clin Cancer Res* 2001;7:3862–3868.
20. Hopfner KP, Hornung V. Molecular mechanisms and cellular functions of cGAS-STING signalling. *Nat Rev Mol Cell Biol* 2020;21:501–521.
21. Benmebarek M-R, Karches CH, Cadilha BL, et al. Killing mechanisms of chimeric antigen receptor (CAR) T Cells. *Int J Mol Sci* 2019;20:1283.
22. Xiong H, Xi Y, Yuan Z, et al. IFN- γ activates the tumor cell-intrinsic STING pathway through the induction of

- DNA damage and cytosolic dsDNA formation. *Oncoimmunology* 2022;11:2044103.
23. Zhang L, Wei X, Wang Z, et al. NF- κ B activation enhances STING signaling by altering microtubule-mediated STING trafficking. *Cell Rep* 2023;42:112185.
 24. Hines JB, Kacew AJ, Sweis RF. The development of STING agonists and emerging results as a cancer immunotherapy. *Curr Oncol Rep* 2023;25:189–199.
 25. Conde E, Vercher E, Soria-Castellano M, et al. Epitope spreading driven by the joint action of CART cells and pharmacological STING stimulation counteracts tumor escape via antigen-loss variants. *J Immunother Cancer* 2021;9:e003351.
 26. Le Naour J, Zitvogel L, Galluzzi L, et al. Trial watch: STING agonists in cancer therapy. *Oncoimmunology* 2020;9:1777624.
 27. Amouzegar A, Chelvanambi M, Filderman JN, et al. STING agonists as cancer therapeutics. *Cancers* 2021;13:2695.
 28. Gulen MF, Koch U, Haag SM, et al. Signalling strength determines proapoptotic functions of STING. *Nat Commun* 2017;8:427.
 29. Dimitri A, Herbst F, Fraietta JA. Engineering the next-generation of CAR T-cells with CRISPR-Cas9 gene editing. *Mol Cancer* 2022;21:78.
 30. Xu N, Palmer DC, Robeson AC, et al. STING agonist promotes CAR T cell trafficking and persistence in breast cancer. *J Exp Med* 2021;218:e20200844.
 31. Smith TT, Moffett HF, Stephan SB, et al. Biopolymers codelivering engineered T cells and STING agonists can eliminate heterogeneous tumors. *J Clin Invest* 2017;127:2176–2191.
 32. Uslu U, Sun L, Castelli S, et al. The STING agonist IMSA101 enhances chimeric antigen receptor T cell function by inducing IL-18 secretion. *Nat Commun* 2024;15:3933.
 33. Su M, Zheng J, Gan L, et al. Second messenger 2'3'-cyclic GMP-AMP (2'3'-cGAMP): synthesis, transmission, and degradation. *Biochem Pharmacol* 2022;198:114934.
 34. Moser JC, Alistar A, Cohen E, et al. 618 Phase 1 clinical trial evaluating the safety, biologic and anti-tumor activity of the novel STING agonist IMSA101 administered both as monotherapy and in combination with PD-(L)1 checkpoint inhibitors. *J Immunother Cancer* 2023;11:A704.
 35. Sivick KE, Desbien AL, Glickman LH, et al. Magnitude of therapeutic STING activation determines CD8⁺ T cell-mediated anti-tumor immunity. *Cell Rep* 2018;25:3074–3085.e5.
 36. Crouse J, Kalinke U, Oxenius A. Regulation of antiviral T cell responses by type I interferons. *Nat Rev Immunol* 2015;15:231–242.
 37. Li B, Jones LL, Geiger TL. IL-6 promotes T cell proliferation and expansion under inflammatory conditions in association with low-level ROR γ t expression. *J Immunol* 2018;201:2934–2946.
 38. Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: co-inhibitory receptors with specialized functions in immune regulation. *Immunity* 2016;44:989–1004.
 39. Fraietta JA, Lacey SF, Orlando EJ, et al. Determinants of response and resistance to CD19 chimeric antigen receptor (CAR) T cell therapy of chronic lymphocytic leukemia. *Nat Med* 2018;24:563–571.
 40. Czaplicka A, Lachota M, Pączek L, et al. Chimeric antigen receptor T cell therapy for pancreatic cancer: a review of current evidence. *Cells* 2024;13:101.
 41. Klampatsa A, Dimou V, Albelda SM. Mesothelin-targeted CAR-T cell therapy for solid tumors. *Exp Opin Biol Ther* 2021;21:473–486.
 42. Nasiri F, Safarzadeh Kozani P, Salem F, et al. Mechanisms of antigen-dependent resistance to chimeric antigen receptor (CAR)-T cell therapies. *Cancer Cell Int* 2025;25:64.
 43. Giavridis T, van der Stegen SJC, Eyquem J, et al. CAR T cell-induced cytokine release syndrome is mediated by macrophages and abated by IL-1 blockade. *Nat Med* 2018;24:731–738.
 44. Norelli M, Camisa B, Barbiera G, et al. Monocyte-derived IL-1 and IL-6 are differentially required for cytokine-release syndrome and neurotoxicity due to CAR T cells. *Nat Med* 2018;24:739–748.

Received March 1, 2025. Accepted January 23, 2026.

Correspondence

Address correspondence to: Veit Hornung, MD, Gene Center and Department of Biochemistry, Ludwig-Maximilians-Universität München, Feodor-Lynen-Strasse 25, 81377 Munich, Germany. e-mail: veit.hornung@lmu.de.

Acknowledgments

We want to thank Zeynep Ünal (Department of Pediatrics, LMU University Hospital) and Luana Messa and Vanessa Wittmann (all Institute of Clinical Pharmacology, LMU University Hospital) for technical assistance in mouse experiments and Maria del Socorro Escobar Lopez (Department of Medicine II, LMU University Hospital) for culturing AsPC-1 cells. We kindly thank Claudia Ludwig and Jochen Rech for technical support in isolation of primary immune cells and CRISPR-Cas9 knockout generation (all Gene Center, LMU Munich) and Britta Pauli for technical assistance in mouse serum analysis (Institute of Laboratory Medicine, LMU University Hospital).

CRedit Authorship Contributions

Ignazio Piseddu, MD (Conceptualization: Equal; Data curation: Supporting; Formal analysis: Equal; Funding acquisition: Supporting; Investigation: Lead; Methodology: Lead; Resources: Supporting; Visualization: Lead; Writing – original draft: Lead; Writing– review & editing: Lead)
 Rebekka Endres (Investigation: Equal; Methodology: Equal; Visualization: Equal; Writing – review & editing: Equal)
 Fabian Lanzl (Investigation: Equal; Methodology: Equal; Visualization: Equal; Writing – review & editing: Equal)
 Linda Hammann, MSc (Investigation: Supporting; Methodology: Supporting; Writing review & editing: Equal)
 Marleen Bérouti, PhD (Investigation: Supporting; Methodology: Supporting; Writing review & editing: Equal)
 Matthias Thaler, MD (Investigation: Supporting; Methodology: Supporting; Writing review & editing: Equal)
 Lisa Fahr, PhD (Investigation: Supporting; Methodology: Supporting; Writing – review & editing: Equal)
 Hannah Fischer, PhD (Investigation: Supporting; Methodology: Supporting; Writing review & editing: Equal)
 Varvara Varlamova, MSc (Investigation: Supporting; Methodology: Supporting; Writing – review & editing: Equal)
 Jan Gärtig, PhD (Investigation: Supporting; Methodology: Supporting; Writing review & editing: Equal)
 Daniel Nixdorf, PhD (Investigation: Supporting; Methodology: Supporting; Writing review & editing: Equal)
 Patrick Layritz (Investigation: Supporting; Methodology: Supporting; Writing review & editing: Equal)
 Charlotte Marx, PhD (Investigation: Supporting; Methodology: Supporting; Writing review & editing: Equal)
 Christine Hörth (Investigation: Supporting; Methodology: Supporting; Writing review & editing: Equal)
 Catharina Witte (Investigation: Supporting; Methodology: Supporting; Writing – review & editing: Equal)
 Alpay Bulut (Investigation: Supporting; Methodology: Supporting; Writing review & editing: Equal)

David Illig, PhD (Investigation: Supporting; Methodology: Supporting; Writing – review & editing: Equal)

Anne Marie Senz, PhD (Methodology: Supporting; Writing – review & editing: Equal)

Lesca Holdt, MD (Investigation: Supporting; Methodology: Supporting; Writing review & editing: Equal)

Ivonne Regel, PhD (Investigation: Supporting; Methodology: Supporting; Writing review & editing: Equal)

Adrian Gottschlich, MD (Investigation: Supporting; Methodology: Supporting; Writing review & editing: Equal)

Marion Subklewe, MD (Supervision: Supporting; Writing – review & editing: Equal)

Julia Mayerle, MD (Conceptualization: Supporting; Funding acquisition: Supporting; Supervision: Equal; Writing – review & editing: Equal)

David Anz, MD (Resources: Supporting; Supervision: Supporting; Writing – review & editing: Equal)

Sebastian Kobold, MD (Resources: Equal; Supervision: Equal; Writing – review & editing: Equal)

Andreas Linder, MD (Conceptualization: Supporting; Methodology: Supporting; Supervision: Supporting; Writing – review & editing: Supporting)

Veit Hornung, MD (Conceptualization: Lead; Formal analysis: Equal; Funding acquisition: Lead; Project administration: Lead; Resources: Lead; Supervision: Lead; Validation: Lead; Writing – original draft: Lead; Writing – review & editing: Lead)

Conflicts of interest

These authors disclose the following: Ignazio Piseddu reports reimbursement of travel expenses and lecture honoraria from Roche and received lecture honoraria from Falk Foundation and third-party funding for scientific research from Novartis. Adrian Gottschlich received research support from Tabby Therapeutics and from Nanogami for work unrelated to the paper. Marion Subklewe has received research funding from Amgen, BMS/Celgene, Gilead/Kite, Janssen, Miltenyi Biotech, Molecular Partners, Novartis, Roche, Seagen, and Takeda; honoraria from AbbVie, Amgen, AvenCell, BMS, CanCell Therapeutics, Gilead, Janssen, Miltenyi Biomedicine, Molecular Partners, Pfizer, and Roche; and was invited as speaker by AstraZeneca, BMS, Gilead/Kite, GSK, Janssen, LAWG, Novartis, Pfizer, Roche, and Springer Healthcare. Sebastian Kobold has received honoraria from Cymab, Plectonic, TCR2 Inc., Miltenyi, Galapagos, Novartis, BMS, and GSK. Sebastian Kobold is an inventor of several patents in the field of immuno-oncology. Sebastian Kobold received license fees from TCR2 Inc and Carina Biotech and received research support from TCR2 Inc, Tabby Therapeutics, Catalym GmbH, Plectonic GmbH, and Arcus Bioscience for work unrelated to the manuscript. The remaining authors disclose no conflicts.

Funding

This work was supported by grants from the Bavarian Cancer Research Center (BZKF) (to Ignazio Piseddu), the *Stiftungen zu Gunsten der Medizinischen Fakultät* (Cluster 1, to Ignazio Piseddu), the Novartis Foundation (InCa prize awarded to Ignazio Piseddu), the ERC (ERC-2020-ADG-101018672 ENGINES, to Veit Hornung), and the Deutsche Forschungsgemeinschaft (German Research Foundation, SFB TRR 338 project-ID: 452881907 and SFB TRR 237 project-ID: 369799452, to Veit Hornung). This study was further supported by the Förderprogramm für Forschung und Lehre der Medizinischen Fakultät der LMU (grant number 1138 to Adrian Gottschlich), the BZKF (Adrian Gottschlich and TANGO to Sebastian Kobold), the DFG (grant number GO 3823/1-1 to Adrian Gottschlich; grant numbers KO5055-2-1 and KO5055/3-1 to Sebastian Kobold; grant number NI 2469/1-1 to Daniel Nixdorf), the international doctoral program “i-Target: immunotargeting of cancer” (funded by the Elite Network of Bavaria; to Sebastian Kobold), the Melanoma Research Alliance (grant number 409510 to Sebastian Kobold), Marie Skłodowska-Curie Training Network for Optimizing Adoptive T Cell Therapy of Cancer (funded by the Horizon 2020 program of the European Union; grant 955575 to Sebastian Kobold), Marie Skłodowska-Curie Training Network for Tracking and Controlling Therapeutic Immune Cells in Cancer (funded by the Horizon program of the European Union; grant 101168810 to Sebastian Kobold), Else Kröner-Fresenius-Stiftung (to Adrian Gottschlich, 2025_EKEA-16 to Ignazio Piseddu, and IOLIN to Sebastian Kobold), German Cancer Aid (AvantCAR.de and grant number 70117182 to Sebastian Kobold, grant number 70115514 to Adrian Gottschlich), Ernst Jung Stiftung (to Sebastian Kobold), Institutional Strategy LMUexcellent of LMU Munich (within the framework of the German Excellence Initiative; to Sebastian Kobold), the Go-Bio-Initiative (to Sebastian Kobold), the m4-Award of the Bavarian Ministry for Economical Affairs (to Sebastian Kobold), Bundesministerium für Bildung und Forschung (to Sebastian Kobold), European Research Council (StG 756017, PoC 101100460, and CoG 101124203 to Sebastian Kobold), by the SFB TRR 338 project-ID: 452881907 (to Sebastian Kobold), Fritz-Bender Foundation (to Sebastian Kobold), Deutsche José Carreras Leukämie Stiftung (to Sebastian Kobold), Hector Foundation (to Sebastian Kobold), Bavarian Research Foundation (BAYCELLATOR to Sebastian Kobold), the Bruno and Helene Jöster Foundation (360° CAR to Sebastian Kobold), the Monika Kutzner Foundation (to Sebastian Kobold), the Rurainski Foundation (to Sebastian Kobold), the Brigitte and Dr Konstanze Wegener Foundation (to Sebastian Kobold), and the DKTK School of Oncology (to Adrian Gottschlich and Sebastian Kobold). David Anz was supported by the German Research Foundation (SFB TRR 355 project-ID: 490846870).

Data Availability

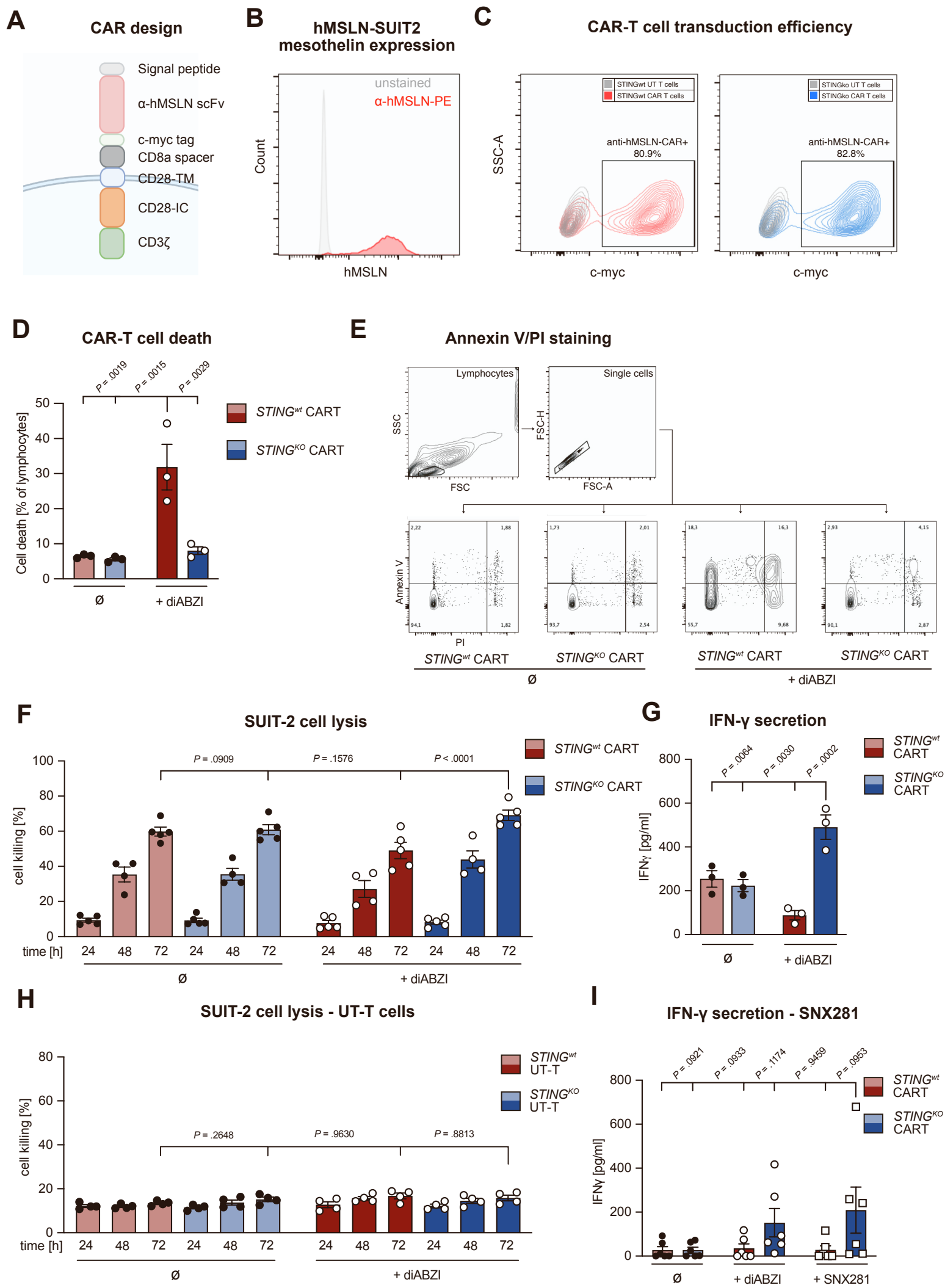
All data are available on request from the corresponding author.

Supplemental information

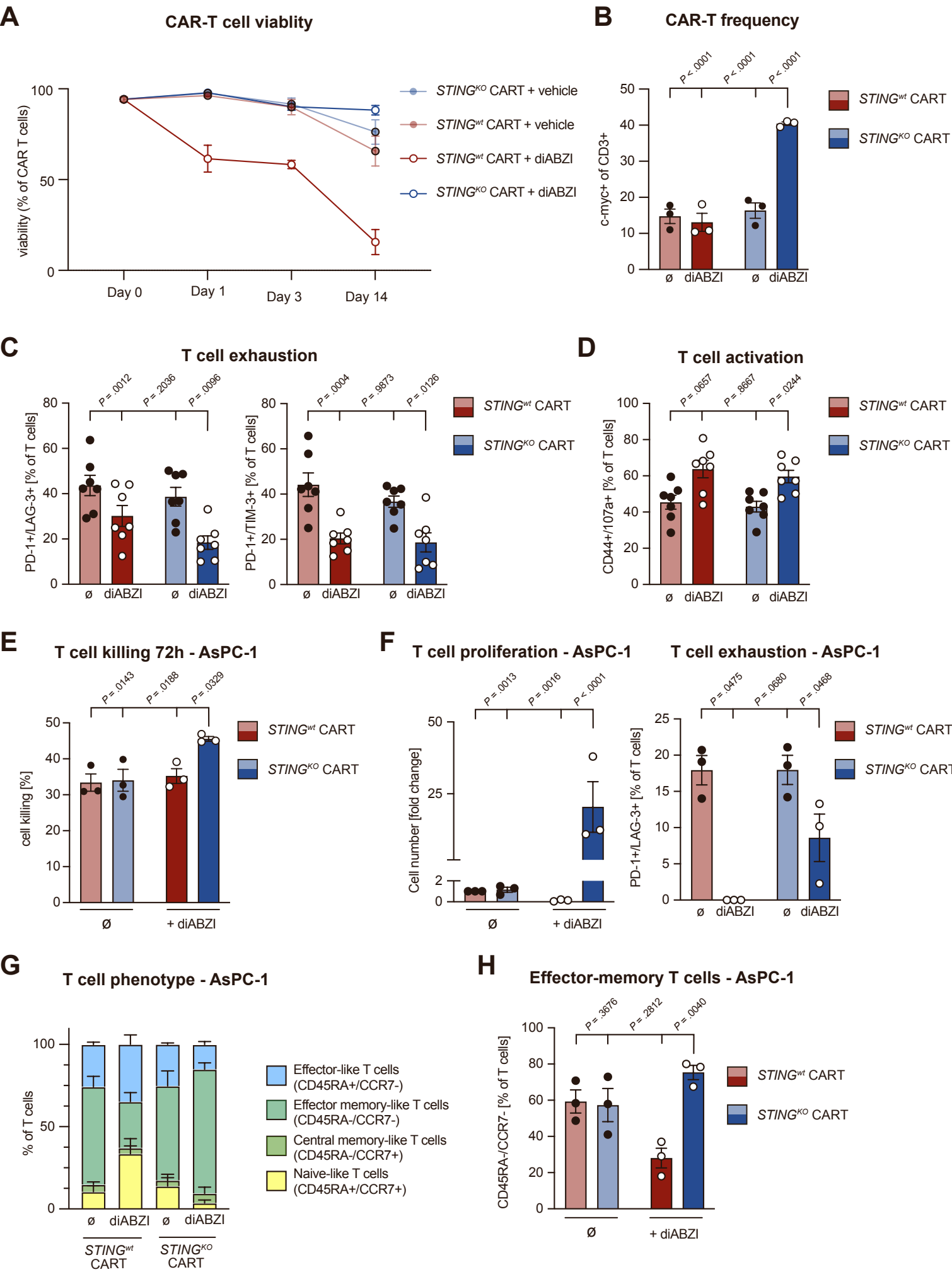
STING Ablation in T Cells Is Required for the Efficacy of STING Agonists in CAR-T Cell Immunotherapy of Pancreatic Cancer

Ignazio Piseddu, Rebekka Endres, Fabian Lanzl, Linda Hammann, Marleen Bérouti, Matthias Thaler, Lisa Fahr, Hannah Fischer, Varvara Varlamova, Jan Gärtig, Daniel Nixdorf, Patrick Layritz, Charlotte Marx, Christine Hörth, Catharina Witte, Alpay Bulut, David Illig, Anne Marie Senz, Lesca Holdt, Ivonne Regel, Adrian Gottschlich, Marion Subklewe, Julia Mayerle, David Anz, Sebastian Kobold, Andreas Linder, and Veit Hornung

Supplementary Figure 1



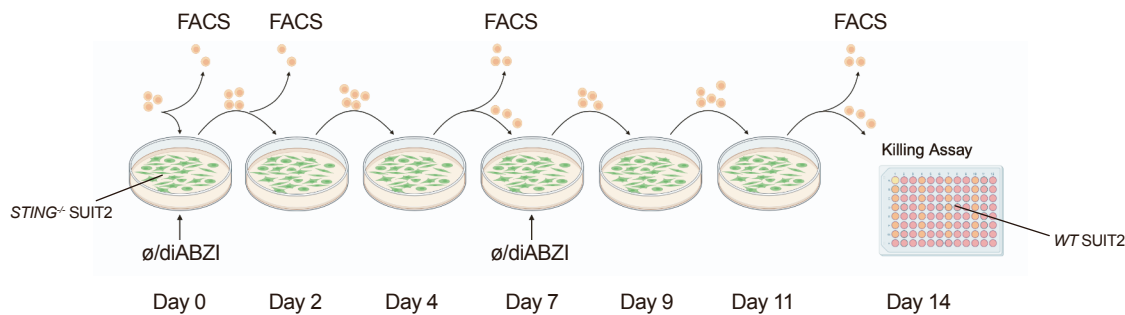
Supplementary Figure 2



Supplementary Figure 3

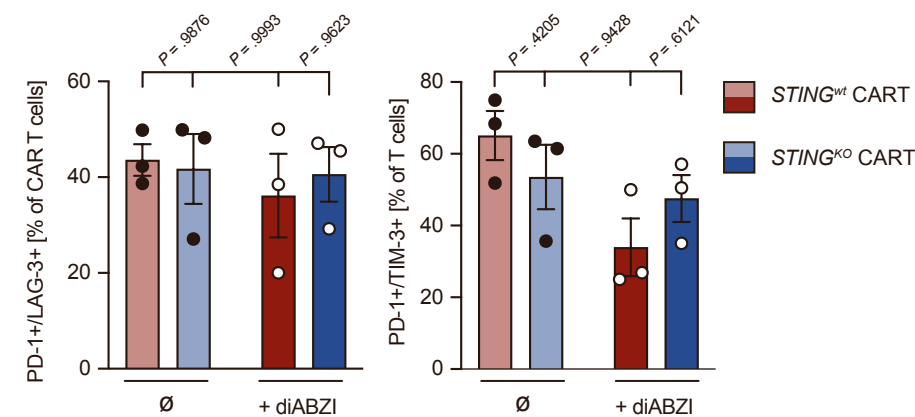
A

CAR-T cell exhaustion protocol



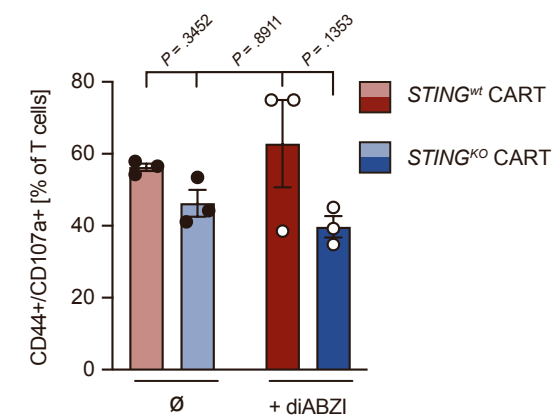
B

T cell exhaustion

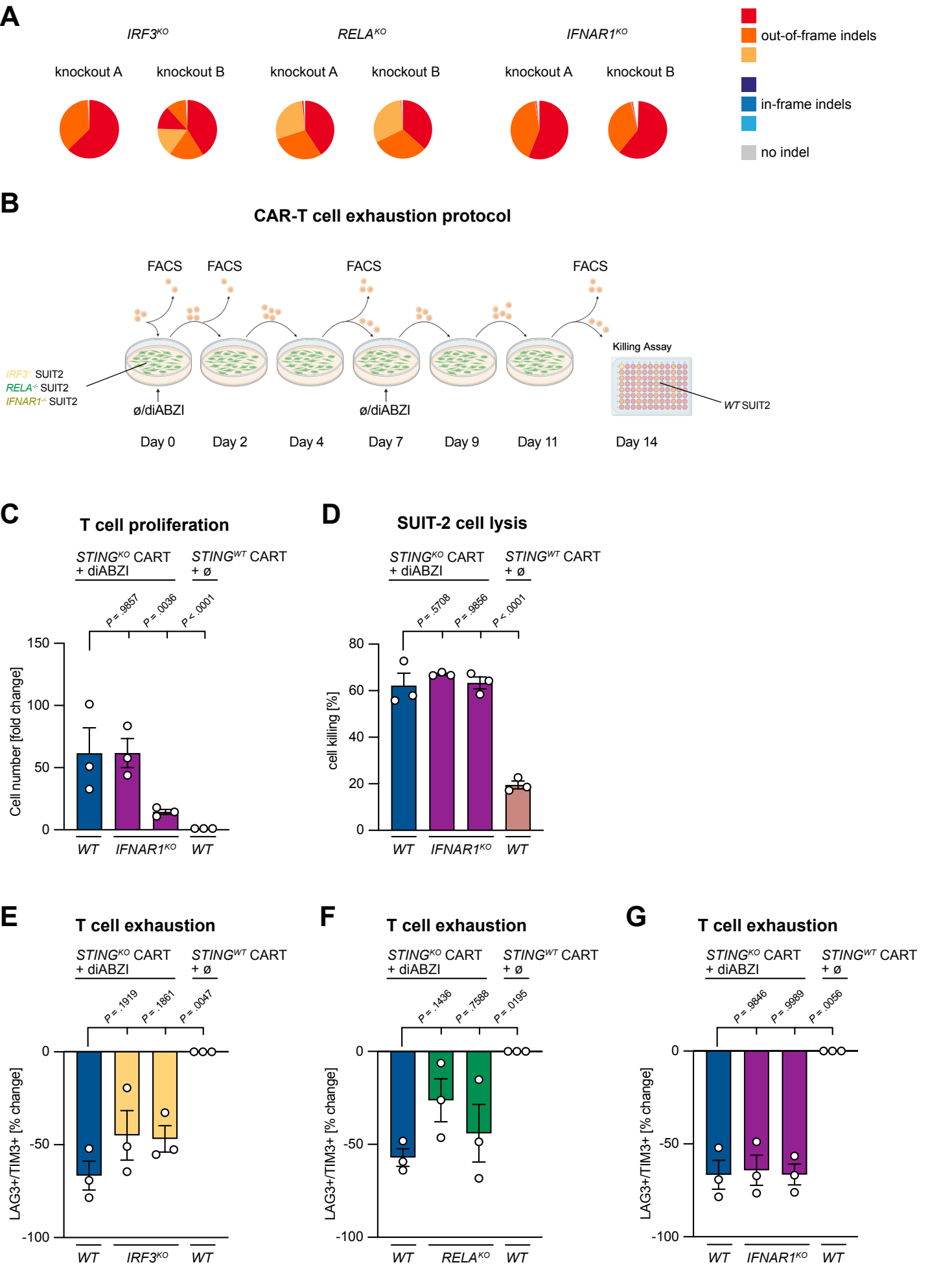


C

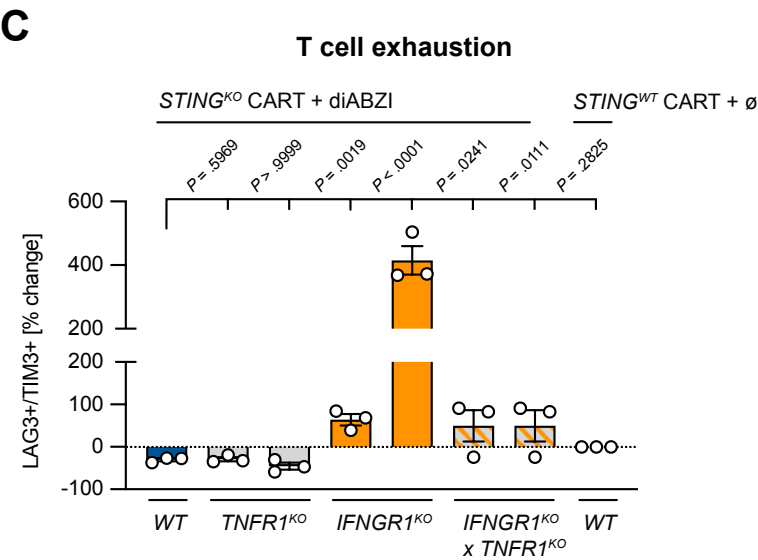
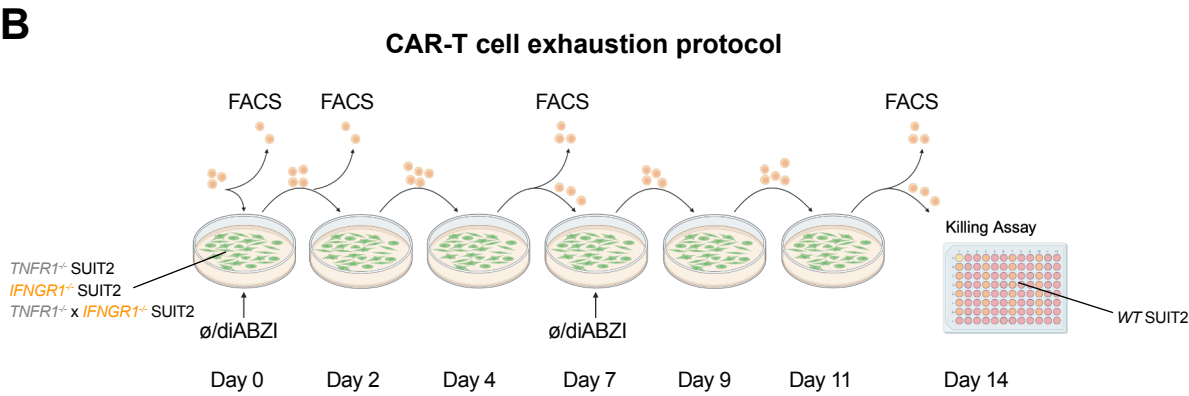
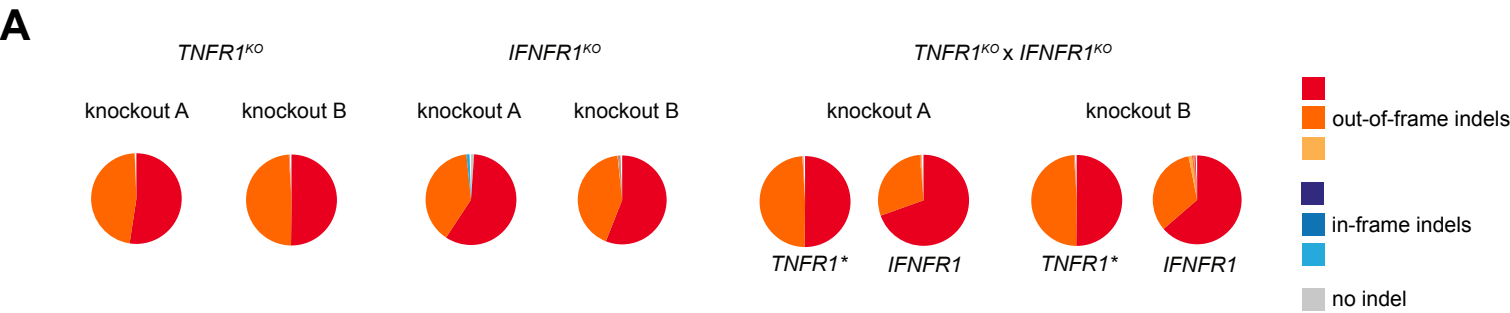
T cell activation



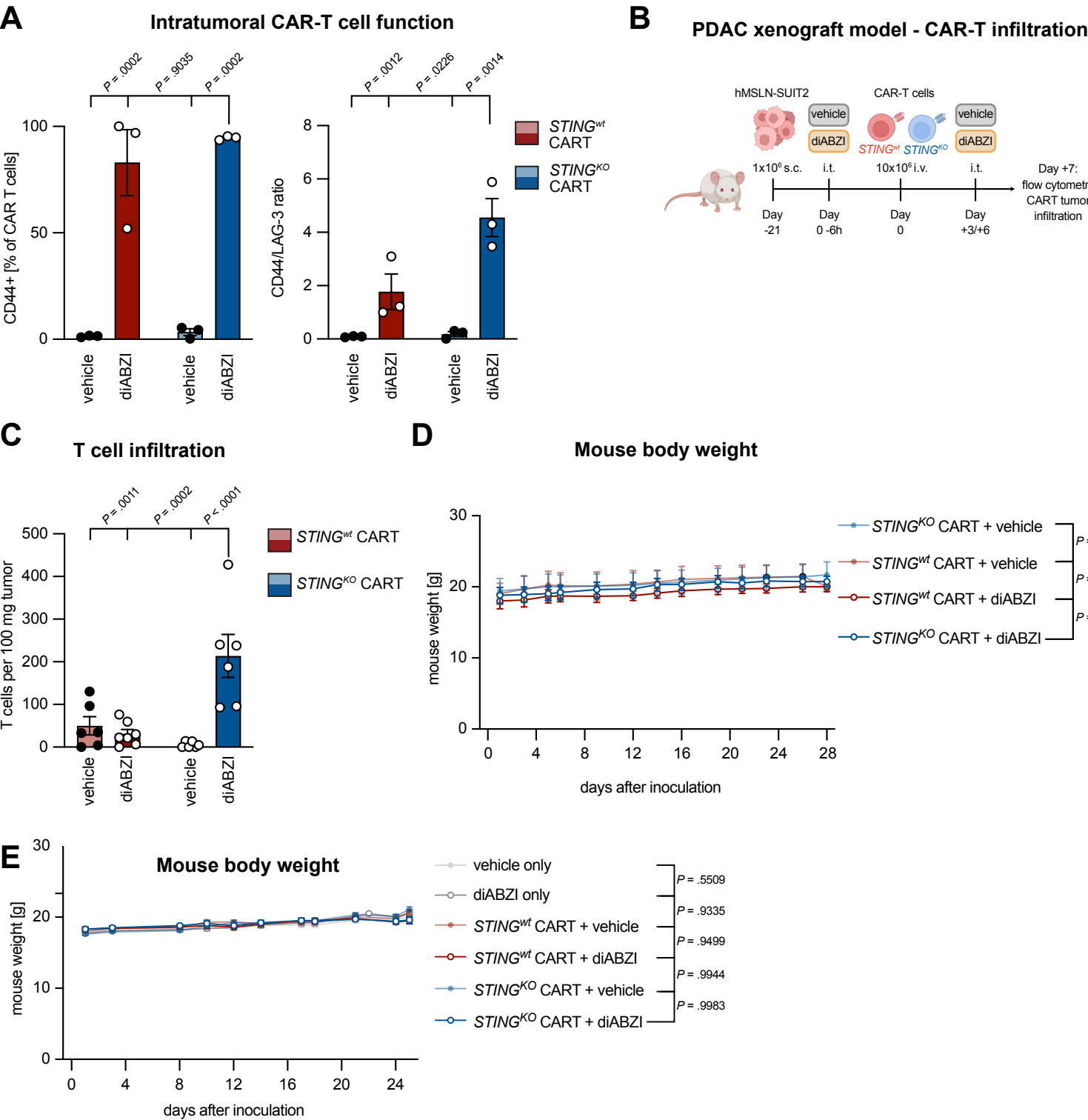
Supplementary Figure 4



Supplementary Figure 5

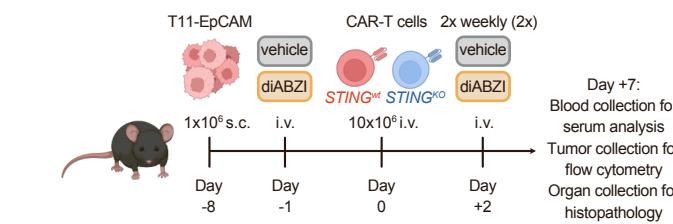


Supplementary Figure 6

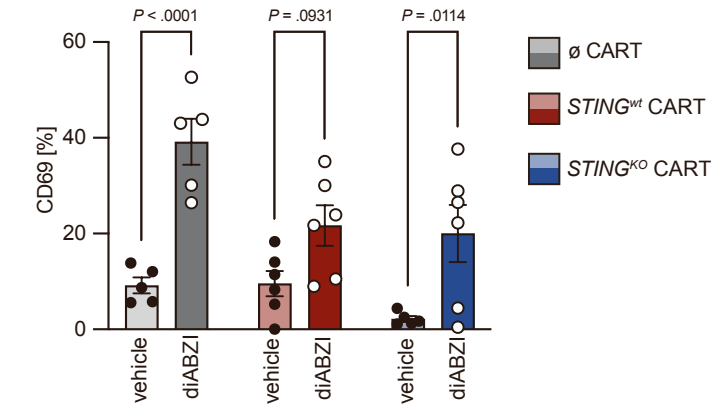


Supplementary Figure 7

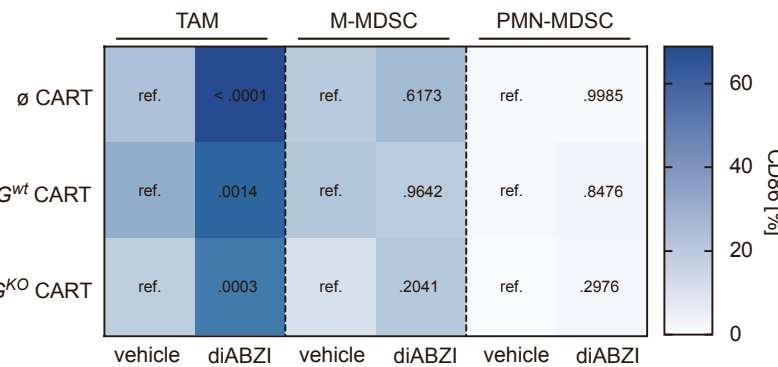
A PDAC syngeneic model - mechanism and safety



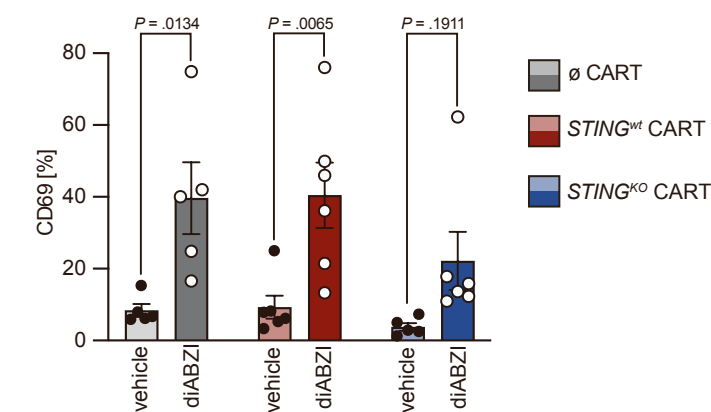
B CD4 T cells



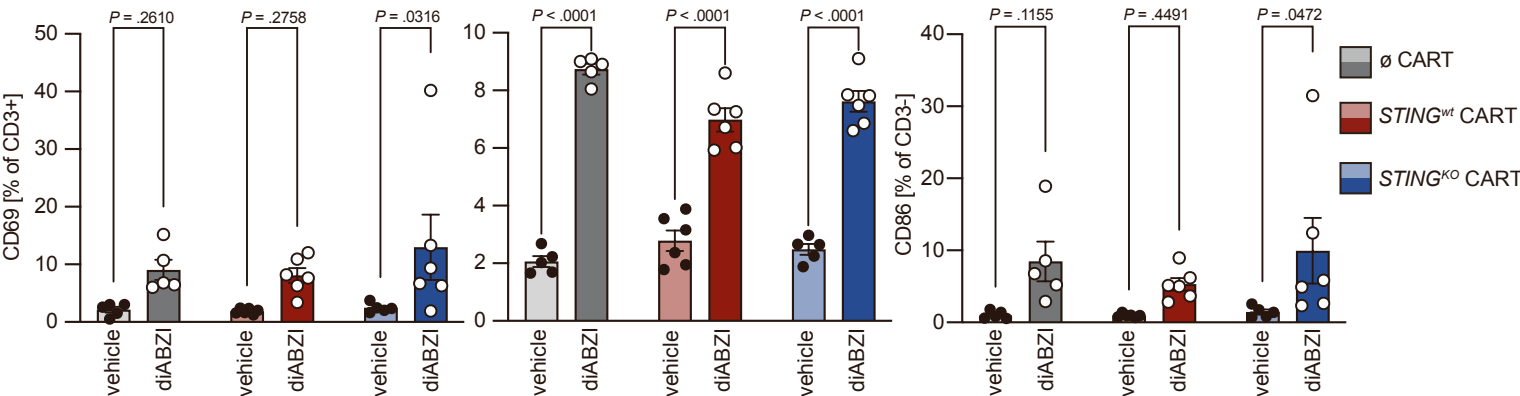
C CD86 expression intratumoral myeloid cells



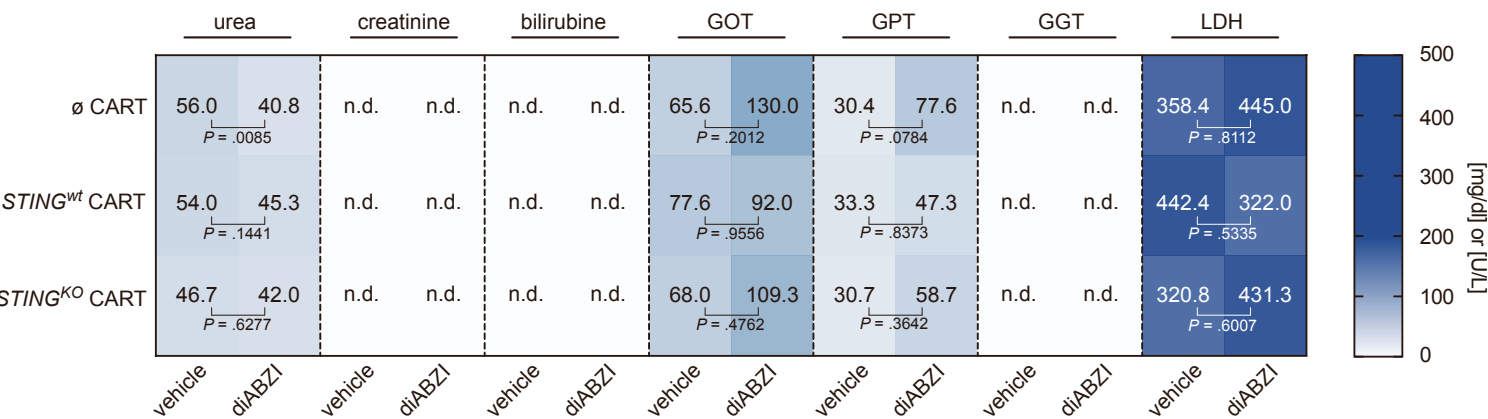
CD8 T cells



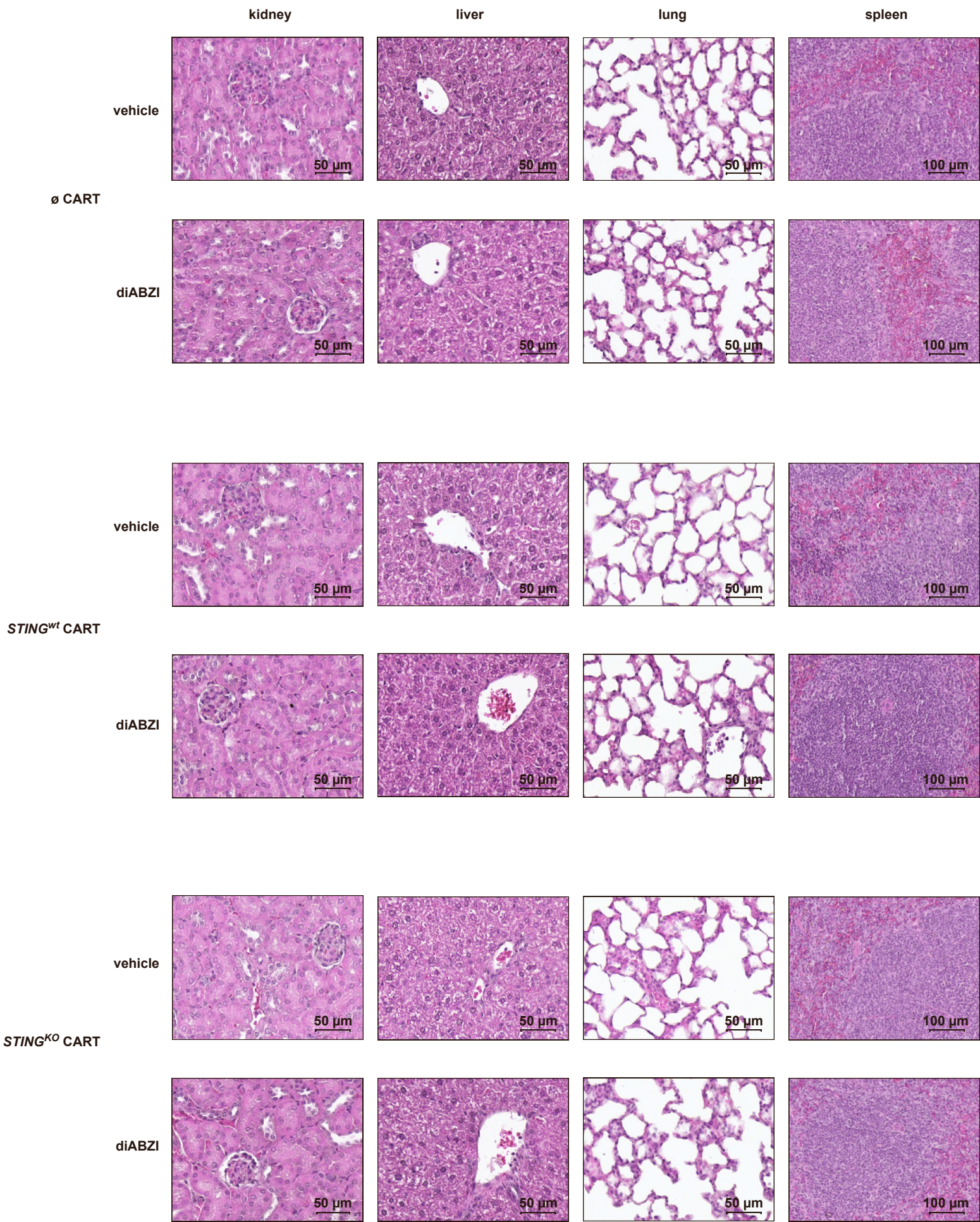
D T cell activation - dLN T cell activation - spleen



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.

References

1. Lesch, S., et al., *T cells armed with C-X-C chemokine receptor type 6 enhance adoptive cell therapy for pancreatic tumours*. Nat Biomed Eng, 2021. **5**(11): p. 1246-1260.
2. Cadilha, B.L., et al., *Combined tumor-directed recruitment and protection from immune suppression enable CAR T cell efficacy in solid tumors*. Sci Adv, 2021. **7**(24).
3. Kuhl, N., et al., *STING agonism turns human T cells into interferon-producing cells but impedes their functionality*. EMBO Rep, 2023. **24**(3): p. e55536.
4. Linder, A., et al., *CARD8 inflammasome activation triggers pyroptosis in human T cells*. Embo j, 2020. **39**(19): p. e105071.

- 224 5. Schmid-Burgk, J.L., et al., *OutKnocker: a web tool for rapid and simple genotyping of*
225 *designer nuclease edited cell lines*. Genome Res, 2014. **24**(10): p. 1719-23.
- 226 6. Ghani, K., et al., *Efficient human hematopoietic cell transduction using RD114- and*
227 *GALV-pseudotyped retroviral vectors produced in suspension and serum-free media*.
228 Hum Gene Ther, 2009. **20**(9): p. 966-74.
229