Supplementary Methods

Multiplex-ISH-IF

Multiplex-ISH-IF was performed by the specialized imaging facility at MGH. Tissue Staining: FFPE tissue sections from 34 mouse xenografts were stained using a Leica Bond RX automated stainer. The staining panel was developed using RNAscope ISH probes (Advanced Cell Diagnostics) with Opal fluorophores (Akoya Biosciences). The panel consisted of 4 RNA probes (Hs-FAP 411978, GFP 409018, mCherry 431208, Hs-CD3E 553978) and one Mesothelin antibody (Abcam ab236546). Tyramide Signal Amplification (TSA) was used to boost fluorophore signal

(Opal 690, Opal 520, Opal 620, Opal 480, and Opal 780). Opal fluorophore concentrations were optimized to balance signal intensity across all channels. A tonsil section and a section from an ASPC1 cultured tumor cell block were stained with each batch as controls. Image Acquisition: Whole slide images of stained slides were acquired at 20x (0.5um) resolution using an Akoya PhenoImager HT multispectral system. For fluorescently labeled samples, exposures were set to avoid saturated tissue pixels. Akoya inForm software was used to separate spectrally adjacent fluorophores and native tissue autofluorescence. The spectral library was created using single-color tonsil slides and unstained mouse tissue. Image Analysis: Cell segmentation and phenotyping was performed within annotated tumor regions using HALO Image Analysis Platform and HALO AI (Indica Labs). Cells within tumor regions were identified using the Dapi nuclear stain. All cells within the tumor region were segmented and counted. Each cell was evaluated for its positivity for the other markers. Areas that contained debris or tissue folds were excluded from the analysis. The FISH-IF v.2.1.5 module was used for cell phenotyping of protein (mesothelin) and RNA ISH markers. Mesothelin positivity was based on signal intensity within the nuclear and cytoplasmic compartments. RNA ISH positivity was based on stain intensity and dot size within a cell, where brighter and larger RNA dots were given more weight. Cell phenotypes were also defined using multiple markers (CD3E+mCherry+FAP-, FAP+mCherry+CD3E-). The analysis app was manually validated for each sample for cell phenotyping quality. Summary tables containing cell phenotype and signal intensity information were exported for analysis across samples.

Mouse Model of Liver Metastasis

NSG mice (NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ; 005557) were purchased from Jackson Laboratory and maintained following the guidelines of the Johns Hopkins University Institutional Animal

Care and Use Committee (IACUC) (Protocol MO22M59). The surgical procedure was conducted in an aseptic operating room. All instruments were autoclaved for sterilization for each mouse, and they were further disinfected with 70% ethanol during the procedure. 1× 10^6 cells/100 µl AsPC-1 (GFP-tagged) and 1× 10^6 cells/100 µl CAF (mCherry-tagged) cells were resuspended in PBS at the ratio 1:1 and kept on ice throughout the experiment. A left subcostal incision was made through the skin and the peritoneum after the mouse was fully anesthetized with isofluorane (2.5%). Afterward, the spleen and adjacent pancreas were pulled out of the body cavity (without completely removing them), allowing for good visualization during cell injection. Medium litigating clips (Horizon, #002200) were used to divide the spleen into two parts, and the upper part was placed back into the peritoneum to avoid contamination. While maintaining the syringe in an upright position, 150 µl PBS followed by 100 µl of cell suspension (1× 10^6 AsPC-1 cells and 1× 10^6 CAF cells) were drawn into the same 26 G x 5/8 syringe. The cells were injected slowly into the exposed hemispleen ensuring the syringe was kept upright at all times, and that the spleen and blood vessels turned white with injection. The splenic vessels were then ligated using a medium Horizon clip, and the injected hemispleen was removed to eliminate any remaining tumor cells. Finally, the peritoneum and skin were closed using 4/0 sutures (Covidien, #GL-191). Mice exhibiting tumor cell leakage during injection were excluded from the study. After the surgery, the mice were randomly divided into five groups. All animal procedures were conducted following the principles of the 3Rs (Replacement, Reduction, Refinement) and were approved by the Institutional Animal Care and Use Committee of Johns Hopkins University.

CAR-T injection**:** On day 14 after surgery, a single dose of 3 × 10^6 CAR-T cells (meso-FAP, meso-CD19, meso CAR, or UTD-T) in 100 ul PBS was administered via lateral tail vein injection. A separate control group received an injection of 100 µl PBS. Each group consisted of

12 mice, and their survival was monitored to assess the efficacy of CAR-T therapy. Of the 60 total mice in the experiment, 5 per group (25 total) were sacrificed on Day 21 (7 days after CAR-T injection) to examine the presence of AsPC-1 cells, CAFs, and CAR-Ts. Livers were collected and divided for fixation in 10% formalin or OCT mounting. Slides were then cut at the Oncology Tissue Services core at the Sidney Kimmel Comprehensive Cancer Center for fluorescent visualization and IHC. The remaining mice (7 per group) were monitored for followed for survival. Mice reaching a survival endpoint, characterized by lethargy, hunched posture, rough hair coat, and dehydration, were euthanized. The examiners conducting the assessments were blinded to the treatment groups.

Fluorescent Imaging: OCT slides were cut and fixed with 4% PFA and mounted with a fluorescence mounting medium (Thermo Fisher Scientific, #00-4959-52). The GFP and mCherry signals were visualized with a fluorescence microscope (Keyence, #BZ-XB10).

Multiplex IHC Imaging**:** Liver samples obtained from an IRB approved protocol at the Johns Hopkins Hospital (IRB00086790) were stained using a multiplex IHC protocol as previously described3. Briefly, 5-mm FFPE tissue section slides were heated at 60 °C for 30 min to remove the wax on the slides. The slides were then de-paraffinized in xylene and rehydrated through graded concentrations of ethanol in water. The de-paraffinized tissue section slides were first stained with hematoxylin (Dako, S3301), followed by whole-slide brightfield scanning using NanoZoomer (Hamamatsu) at the Johns Hopkins Sidney Kimmel Comprehensive Cancer Center Tumor Microenvironment Core. Prior to sequential multiple iterative cycles of IHC staining, the slides underwent endogenous peroxidase blocking (Vector Laboratories, SP-6000-100) and heat-induced antigen retrieval with citrate buffer pH 6.0) (BioGenex, HK080-9K).

Detailed information on the antibodies used (including concentration, incubation time, the concentration of primary and secondary antibodies, the horseradish peroxidase (HRP)-conjugated polymer (Nichirei Biosciences Inc.), and the aminoethyl carbazole (AEC) (Vector Laboratories, SK-4200) reaction time for chromogenic detection) are summarized in Table A. The staining conditions for FAP were developed and optimized as described in Blair et al. Multiplex IHC images were visualized and fused using Halo (v3.6).

Table A

	Cycle1	Cycle2	Cycle3	Cycle4	Cycle5
Primary Ab	**Hematoxylin**	**FAP**	**CD3**	**EPcam**	**CSF1R**
Clone/Product #	S3301	EPR20021 (ab207178)	Sp7 (RM9107S1)	E144 (ab32392)	SP211 (ab183316)
Vendor	Dako	Abcam	Thermo Science	Abcam	Abcam
Concentration	N/A	1/200	1/150	1:500	1:150
Reaction	1 min	1h	30 min	60 min	30min
Secondary Ab Reaction		Anti-rabbit	Anti-rabbit	Anti-rabbit	Anti-rabbit
		RT, 30 min	RT, 30 min	RT, 30 min	RT, 30 min
AEC reaction		20 min	100 min	25 min	60min

Secondary Ab:	ImmPRESS® HRP Goat Anti-Rabbit IgG Polymer Detection Kit, Peroxidase (MP-7451)

Immunohistochemistry: Murine livers were formalin fixed paraffin-embedded and sectioned at 5µm thickness. Paraffin-embedded sections were rehydrated through a series of xylene and different concentrations of alcohol, which was followed with a rinse in water and washing in PBS. Slides were put with an antigen retrieval buffer (3300, Vector Labs) and steamed for 20 min. Slides were then blocked in a peroxide blocking buffer (ab64218, Abcam) for 15 min, followed by protein block (ab64226, Abcam) for 5 min, and were incubated with the primary antibody of interest; (Abcam: FAP ab207178; Cell Signaling: GFP 2555S, CD-3 85061S), which was prepared in antibody diluent (S0809, Dako). Slides were put in a humidified chamber at 4 °C overnight. Samples were washed with PBS and incubated in biotinylated anti-rabbit (ab64256 Abcam), followed by streptavidin–HRP solution at room temperature for 20 min (ab64269, Abcam). Samples were then washed with PBS and incubated with AEC (3-amino-9-ethylcarbazole) chromogen or DAB for CD3 for the appropriate amount of time after optimization (SK-4200, Vector Laboratories). Slides were then washed with water and incubated in hematoxylin (abcam, ab220365) for 10 seconds, rinsed with water, and mounted in VECTASHIELD PLUS (H-1900, Vector Laboratories). Slides were imaged using a Hamamatsu NanoZoomer digital slide scanner at 20x magnification using the associated NDP.toolkit slide processing software. Slides were analyzed for positivity with the HALO analysis software and quantified using Prism V9.5.0.

IncuCyte Cytotoxicity Analysis

The average green area was recorded by IncuCyte as an indicator for remaining tumor cells. CAF and AsPC-1 cells were seeded at low confluency in a 9:1 ratio the day prior to the addition of CAR^{TEAM} or UTD allowing CAF and AsPC-1 to growth during the experiment. CAR^{TEAM} or UTD

were added in a 1:1 E:T ratio. AsPC-1 cells (green) were measured over time using 2-4 images per well per hour. Normalized AsPC-1 cell area was determined as the following; AsPC-1 (target cell) total green object area at each time point / AsPC-1 (target cell) total green object area at CAR^{TEAM} addition (time 0).

Preparation and microfluidic culture of patient-derived organotypic tumor spheroids (PDOTS). Tumor samples were collected from patients and analyzed according to Dana-Farber/Harvard Cancer Center IRB-approved protocols. These studies were conducted according to the Declaration of Helsinki and approved by the DF/HCC IRB. PDOTS were prepared and cultured, as previously described (35, 82). PDOTS were generated from a patient with a tumor node metastasis (TNM) grading of ypT3 N1, representing a borderline resectable tumor with regional lymph node metastasis. Before surgery, this patient was pre-treated with the neo-adjuvant chemotherapeutic regimen FOLFIRINOX followed by capecitabine with radiotherapy. A fresh tumor specimen was collected during the surgical resection. An explanted pancreatic ductal adenocarcinoma (PDAC) specimen was received in media (DMEM) on ice and minced using a sterile forceps and scalpel (Day 0). The minced tumor specimen was resuspended in RPMI with 10% FBS, 1% pen-strep, 100 U/mL type IV collagenase, and 15 mM HEPES. After 30 min, an equal volume of media was added to the minced PDAC tumor specimen. The cell suspension was pelleted, resuspended in fresh media, and sequentially passed over 100 mm and 40 mm filters to obtain S1 (>100 μm), S2 (40-100 μm), and S3 (<40 μm) spheroid fractions, which were transferred to ultra-low attachment tissue culture plates. The S2 fraction was pelleted and resuspended in type I rat tail collagen (Corning) at a concentration of 1.7 mg/mL following the addition of 10x PBS with phenol red with pH adjusted using NaOH (final pH 7.0–7.5). The spheroid-collagen mixture

was injected into the center gel region of the 3D microfluidic culture device (10 μL/device). After incubation (30 min, 37°C), collagen hydrogels containing PDOTS were hydrated with media (250 μL). S1 and S3 fractions were cryopreserved. After confirming PDOTS growth in the AIM microfluidic devices by light microscopy, CAR T-cells were thawed in R10 media containing IL-2 and cultured overnight via the side channel. On Day 2, CAR-T-cells were counted and, based on % viable cells, were added to the side/media channel at the desired effector:target (E:T) ratio of 1:3. After an additional three days (Day 5), conditioned media was collected and banked for cytokine profiling. PDOTS were stained and imaged for viability assessment. The optimal E:T ratio and cultured conditions were determined using cell line-derived tumor spheroids using the AsPC-1 cell line.

Viability assessment of PDOTS.

Dual label fluorescence live/dead staining was performed using Hoechst/ propidium iodide (Ho/PI) staining solution (Nexcelom, CSK-V0005), as previously described (35). Following incubation with Ho/PI (20 min, 37°C, 5% CO2), images were obtained. Image capture and analysis are performed using a Nikon Eclipse NiE fluorescence microscope equipped with Z-stack (Prior), motorized stage (ProScan), and ZYLA5.5 sCMOS Camera (Andor). Live and dead cell quantitation was performed by measuring total cell area of each dye using NIS-Elements AR software package. Percent change in cell viability was generated using raw fluorescence data for given treatments relative to control conditions.

Secreted Cytokine Profiling of PDOTS.

Analysis of secreted cytokines from PDOTS was performed utilizing a bead-based immunoassay approach using the MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel (Cat# HCYTMAG-60K-PX30). Conditioned media (25 µL) from PDOTS were assayed neat. Concentration levels (pg/mL) of each analyte were derived from 5-parameter curve fitting models using MAGPIX software. Lower and upper limits of quantitation (LLOQ/ULOQ) were imputed from standard curves. Fold changes relative to control samples were calculated and plotted as $\log_2$ fold change (L2FC).

Patient-derived Organoids

Patient sample acquisition and organoid and CAF line generation: Patients with PDAC undergoing surgical resection were enrolled in IRB-approved tissue acquisition protocols at Johns Hopkins Hospital (NCT03563248). Patient-derived organoids (PDOs) were generated from patient surgical specimens following a combination of mechanical and enzymatic dissociation as previously described (9). Organoid lines were maintained in Matrigel (Corning, 356234) with Human Complete Feeding Media. For organoid passaging, media was aspirated and then Matrigel domes were resuspended in Cell Recovery Solution (Corning, 354253) and incubated on ice at 4°C for 45 minutes for Matrigel depolymerization. Cells were then pelleted and washed in human organoid wash media (Advanced DMEM/F12, 10mM HEPES, 1x GlutaMAX, 100µg/mL Primocin, 0.1% BSA) prior to pelleting again. Cell pellets were split 1:2 and replated in Matrigel in new 24 well plates and placed in the incubator for 10 minutes to allow Matrigel to harden. Human Complete Feeding Media was added on top of Matrigel domes and plates were returned to the incubator for further expansion. CAFs were extracted from surgical resection specimens after remnant tissue was washed twice with human organoid wash media with centrifugation between

washes and strained through a 70µm cell strainer. Cells were plated into 1 well of a 6 well plate and allowed to expand before further cell passaging and expansion. CAFs were expanded by trypsinization and expanded at a rate of 1:2 in CAF media (RPMI + 10% FBS + 1% Pen/Strep + 0.1% Amphotericin B). RPMI (Fisher 11-875-085), Pen/Strep (Gibco 5140122), Amphotericin B (Sigma A2942). PDOs and CAFs were mycoplasma tested and underwent lineage verification using short tandem repeat (STR) assessment though the Johns Hopkins University Genetic Resources Core Facility.

Co-culture setup and cell acquisition: CAR-T-cells were combined with PDOs and CAFs at a ratio of 1:3:12 organoids to CAFs to T-cells. Co-cultured cells were resuspend in 25% Matrigel (Corning, 356234) supplemented with 75% Human Complete Feeding Media+10% Human A/B serum and plated in triplicate in 24-well tissue culture dishes. Matrigel domes were allowed to harden at 37°C for 1 hour before 500ul of Human Complete Media + 10% human A/B serum was added to each well. CAR T-cell alone conditions were supplemented with 100IU IL-2 in media. Cells were maintained in co-culture for a total of 5 days with an interim harvest at day 2. To extract T-cells from co-culture, supernatant was aspirated, and each dome was digested with a 2mg/mL collagenase IV for 45min at 37°C. Wash media was added to each well to quench collagenase digest and each well was transferred to a 96-well deep well plate to collect. The plate was centrifuged for 5min at 1500 RPM, supernatant was aspirated, and cells were resuspended in wash media. Centrifugation and supernatant aspiration were then repeated. Cell pellets were resuspended in TrypLE Express (ThermoFisher Scientific, 12604013) following manufacturer instructions to dissociate co-cultured cells. Cells were resuspended in MACS buffer (PBS + 2ml 5 mM EDTA + 1% Fetal bovine serum) and transferred to a 96 well U bottom plate and centrifuged at 1800 RPM for 2 minutes and resuspended for staining for flow cytometry. All antibodies except

FAP were purchased from Biolegend. Cells were resuspended in PBS + Zombie NIR (catalog no. 423106; dilution 1:1000) + Human TruStain FcX (catalog no. 422302; dilution 1:100) for 10 minutes at room temperature in the dark. Cells were quenched with MACS buffer, centrifuged, and then resuspended in surface stain for 20 minutes on ice at 4°C in the dark;, PE FAP (R&D catalog no. FAB3715P; dilution 1:75), APC EpCAM (catalog no. 324208;dilution 1:200), PE/Cy7 LAG-3 (catalog no. 369309; dilution 1:200), AF700 CD3 (catalog no. 300323;dilution 1:400), PerCP/Cy5.5 TIM-3 (catalog no. 345016; dilution 1:100), BV785 PD-1 (catalog no 367431;dilution 1:100), BV421 CD69 (catalog no. 310929; dilution 1:200). Cells were washed twice in MACS buffer. Flow cytometry was performed on the Beckman Coulter Cytoflex and analyzed by FlowJo version 10.8.0.

Co-culture imaging: Cell combinations were plated in a 24-well tissue culture dishes in the co-culture combinations and ratios described above. After plating, cells were visualized using a Nikon ECLIPSE Ti2 microscope at 20x magnification. Images were acquired using transillumination for complete cell composition and RFP to visualize T-cells containing the mCherry reporter. Transillumination and RFP images were merged using the NIS-Elements AR Software (Version 5.21.03). Images were acquired on Day 0 after plating 5-day flow cytometry experiments with subsequent images obtained at the Day 2 and Day 5 timepoints prior to cell collection for flow cytometry evaluation.

For western blotting, FAP and mesothelin screening was completed across multiple CAF and organoid lines. Lysates were collected using RIPA (ThermoFisher) buffer with 100x Protease Inhibitor Cocktail (ThermoFisher). Protein was quantified using the Pierce BCA Protein Assay (ThermoFisher, 23227) with readout on a SpectraMax M3 (Molecular Devices). 20µg of protein was then prepared with 4x Protein Sample Loading Buffer (Li-COR, 928-40004) and 20x

Reducing Agent (BioRad, 161-0792) and boiled at 95°C for 5 minutes. Samples were loaded into a NuPage 4-12% Bis-Tris 1.5mm Mini Protein Gel (Fisher Scientific) with a coordinating prestained protein ladder (Chameleon Duo NIR, Li-COR). Gels were run for 30 minutes at 200V and then transferred to a nitrocellulose membrane using the iBLOT2 Dry Blotting System (ThermoFisher) and iBlot 2 Transfer Stacks (ThermoFisher, IB23001). Following transfer, membrane was blocked at room temperature for 1 hour using the Intercept (TBS) Blocking Buffer (Li-COR, 927-60001). Membranes were then placed in primary antibody (Cell Signaling Technology, FAP: 66562S; mesothelin: 99966S; GAPDH: 97166S), diluted at 1:1000 overnight at 4°C. Membranes were then washed for 5 minutes 4 times with TBS-T. Secondary antibodies (Li-COR, IRDye 800CW Donkey anti-Rabbit 926-32213; IRDye 680RD Donkey anti-mouse, 926-68072) were then added at 1:5000 for 1 hour at room temperature. Membranes were then washed again for 5 minutes 4 times with TBS-T and then stored for imaging in TBS. Images were obtained on the Odyssey CLx Imager (Li-COR).

VITAL Assay: Target cells (JHH 317 PDO) were isolated after media was aspirated and then Matrigel domes were resuspended in Cell Recovery Solution (Corning, 354253) and incubated on ice at 4°C for 45 minutes for Matrigel depolymerization. Cells were pelleted at 1500 RPM for 5 minutes and washed in human organoid wash media (Advanced DMEM/F12, 10mM HEPES, 1x GlutaMAX, 100µg/mL Primocin, 0.1% BSA) prior to pelleting again at 1500 RPM for 5 minutes. Cell pellets were resuspended in TypLE express following manufacturer instructions to dissociate organoids into single cells. Wash media was added to quench the reaction and cells were pelleted at 1500 RPM for 5 minutes. Cells were resuspended in PBS, counted and centrifuged to pellet at 1500 RPM for 5 minutes. Cells were resuspended in 1:1000 Cell Trace Violet (ThermoFisher C34557) in PBS at a concentration of 10X10^6 cells/mL. Cells were then incubated

at 37C for 20 min in the dark to allow for dye incorporation. Reaction was quenched with wash media and cells were centrifuged at 1500 RPM and recounted. UTD CAR T cells were used as the nontarget control. UTD were harvested after being rested for 1 day in T cell media (RPMI 1640 + 10% Human A/B serum, + 1% Pen/strep) + 100IU/mL IL-2. UTD were centrifuged to pellet at 1500 RPM for 5 min and resuspended in PBS and counted. Cells were then centrifuged again to pellet at 1500 RPM for 5 min and resuspended in 1:2000 Cell Trace CFSE proliferation dye (ThermoFisher C34554) in PBS and placed in the incubator at 37C for 20 minutes in the dark. Reaction was quenched with wash media and cells were centrifuged at 1500 RPM and recounted. Target and nontarget control cells were plated in 96 well U bottom plate at a 1:1 ratio in every condition (cell count = 20,000 cells target and 20,000 cells nontarget control). mCherry transduced CART cells were sorted for mCherry+ pure populations using the BD fusion sorter. mCherry + cells were plated at ratios of 0.5:1:1, 1:1:1, 3:1:1, 5:1:1, 10:1:1 CAR T effector : Target : nontarget and placed in the incubator at 37C for 1 day. To harvest cells, the plate was centrifuged at 1500 RPM for 5 min and cells were washed 1X with Macs buffer (500mL PBS + 5mL FBS + 2mL 0.5mM EDTA) and respun. Cells were resuspended in PBS + human TruStain FcX (catalog no. 422302; dilution 1:100) + Zombie Nir IR (catalog no. 423106; dilution 1:1000) for 10min at room temperature in the dark. Cells were washed 1X with Macs buffer and spun down at 1500 RPM for 5 min. Cells were resuspended in Macs buffer and acquired. Flow cytometry was performed on the Beckman Coulter Cytoflex and analyzed by FlowJo version 10.8.0. Variation in proportion of cells in different populations with different fluorescence was assessed in wells that did not contain any effector cells (0:1:1 condition). All data were adjusted using:

Adjusted % survival = 100% survival mean %survival in absence of effectors. Percent specific lysis was calculated by: % specific lysis = 100 – adjusted % survival.