SUPPLEMENTARY MATERIALS AND METHODS

Study population. This study focused on patients enrolled in the phase 2 of the H9H-MC-JBAJ clinical trial, a global, two-part investigation assessing the oral administration of galunisertib in combination with gemcitabine. The initial phase of the study consisted of a multicenter, open-label, non-randomized dose-escalation trial. The subsequent phase employed a randomized, double-blind, two-arm design in a 2:1 ratio, comparing galunisertib plus gemcitabine to gemcitabine with placebo.

Eligible participants were required to meet the following criteria: (1) a histologically or cytologically confirmed diagnosis of pancreatic ductal adenocarcinoma (PDAC), either locally advanced (Stage II or III) or metastatic (Stage IV), for which surgical resection with curative intent was not feasible. Patients who had previously undergone curative surgery for PDAC could be included upon documented disease progression. Those who had received adjuvant treatment with gemcitabine-based chemotherapy or chemoradiotherapy were eligible if such therapy had been completed at least three months prior to enrollment; (2) the presence of measurable or non-measurable disease, as defined by RECIST version 1.1; (3) age ≥18 years, regardless of sex; (4) adequate baseline organ function, including hematologic (absolute neutrophil count ≥1.5 × 10^9/L, platelet count ≥100 × 10^9/L, hemoglobin ≥9 g/dL), hepatic (bilirubin ≤1.5 × upper limit of normal [ULN], AST, ALT, and ALP ≤2.5 × ULN), and renal (serum creatinine ≤1.5 × ULN) parameters; (5) an Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 2; (6) absence of moderate to severe cardiovascular disease; and (7) exclusion of individuals with endocrine tumors of the pancreas or ampullary carcinomas. All procedures involving human participants, tissues, or data were conducted according to the principles of good clinical practice, applicable laws and regulations, the Council for International Organizations of Medical Sciences International Ethical Guidelines, and the Declaration of Helsinki. The local ethical review board approved the study. Written informed consent was received before participation.

Compounds and treatments. Recombinant Angiogenin (rAng) (Cat. No.CSB-EP001703MO, Cusabio Technology LLC) and recombinant Tnfα (rTnfα) (Cat. No. 410-MT, R&D System) were used for 48h at 250 ng/mL (rAng) or 200 ng/mL (rTnfα), respectively. Gemcitabine (Cat. no.S1149), etanercept (Cat. no. HY108847), galunisertib (Cat. No. S2230), Cytochalasin D (Cat. no. HY-N6682), Rhosin hydrochloride (Cat. no. HY-12646), and ETH-1864 (Cat. No. HY-16659) were purchased from MedChemExpress. For in vitro experiments, all compounds were dissolved in 100% dimethyl sulfoxide (DMSO) and used at the following concentrations: 0.78nM-200nM gemcitabine, 5μM galunisertib, 5μg/ml etanercept, 2.5μM cytochalasin D, 10μM rhosin hydrochloride or 15μM ETH-1864. For in vivo treatments, gemcitabine was resuspended in saline solution and administered intraperitoneally at 75 mg/Kg concentration once/week, while galunisertib was resuspended in hydroxyethylcellulose (HEC) (0.25%) and administered via oral gavage at 100 mg/kg concentration twice/day.

Cell cultures. Pancreatic cancer cells were kindly provided by Dr. Paola Cappello (University of Turin, Italy) and Prof. Vincenzo Bronte (University of Verona). Cells were isolated by tumors developed in LSL-$Kras^{G12D/+}$; $p53^{R172H/+}$; $PdxCre^{tg/+}$ (KPC) or LSL-$Kras^{G12D/+}$; $PdxCre^{tg/+}$ (KC) mice. RAW 264.7 cells were purchased from ATCC. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Euroclone, Milan, Italy) containing 10% fetal bovine serum (FBS) (Euroclone), 1% penicillin-streptomycin (PS) solution (Gibco) and 1% Glutamine (glu) (Gibco) at 37°C in a 5% CO_2 incubator. All cells were regularly tested by PCR to exclude Mycoplasma contamination.

Bone-marrow derived macrophages (BMDM) were isolated by flushing femurs of adult C57BL6/J mice and culturing the resulting cells in RPMI containing 10% FBS, 1% PS and 1% glu supplemented with 50ng/mL recombinant m-CSF (Miltenyi Biotec) for 7 days, and then polarized for 48h with IFN-γ (50 ng/mL, Miltenyi Biotec) or Tgfβ (20 ng/mL, Bio-techne).

Co-culture experiments were performed using a hydrophobic silicon barrier in ø100 mm Petri dishes. $1x10^6$ BMDM or $1x10^6$ RAW264.7 were plated in half of the dish with $6x10^5$ PDAC cells, and let to attach overnight. After cutting the barrier, cells were allowed to communicate for 48h in low serum conditions (DMEM 1% FBS) in the absence or presence of treatments.

Quantification of the effect of treatments. To evaluate the response of PDAC cell lines to the combination of gemcitabine plus galunisertib, the conditioned medium (CM) from co-cultures was filtered and used for treatments. $1x10^3$ cells were seeded in 96-well plates and treated for 72h with the CM containing DMSO or increasing concentrations of gemcitabine, in presence or absence of 5μM galunisertib. When indicated, 5μg/ml etanercept or 200 ng/ml rTnfα were combined. Crystal violet was used to measure cell viability using a plate reader (Victor X4, PerkinElmer). Mean EC50 values and 95% CIs were calculated using GraphPad Prism software (GraphPad Software Inc).

Plasmids and lentiviral production. Lentiviruses for gene knockdown were produced in HEK-293T cells by co-transfecting lentiviral vector, dR8.74 packaging plasmid (Addgene #22036) and pMD2.G envelope plasmid (Addgene #12259). shRNA vectors used were: pLKO.1-puro (scramble, LV-c; Addgene #8453), pLKO.1-puro-shAng.1 (targeting sequence 5′-AGGATGACTCCAGGTACACAA-3′ and pLKO.1-puro-shAng.2 (targeting sequence 5′-TGATGAAGAGAAGAAGCCTAA-3′). When unspecified, gene silencing was performed by co-infection of PDAC cells with both Ang-targeting lentiviruses. Lentiviruses for gene overexpression were produced in HEK-293T by co- transfection of pLenti-GIII-CMV vector (Cat. No. 49645064, Applied Biological Materials Inc.) or pLenti-GIII-CMV-Ang (Cat. No. 11885064, Applied Biological Materials Inc.), with dR8.74 packaging (Addgene #22036) and pMD2.G envelope vectors (Addgene #12259).

Immunofluorescence. 15.000 BMDM cells were seeded on positively charged glass slides (Thermo Fisher Scientific), fixed for 30 min with 4% paraformaldehyde (PFA) (Thermo Fisher Scientific) and 4% sucrose (Sigma) solution, washed with PBS and permeabilized with PBS/BSA 1%/Triton 0.3% solution for 10 min. Cell cytoskeleton F-actin was labeled with Alexa Fluor® 555 Phalloidin (#8953, Cell Signaling Technology, Danvers, MA, USA) for 15 min. Next, BMDM were washed with PBS, cell nuclei stained with DAPI (#4083 Cell Signaling Technology, Danvers, MA, USA), and slides closed with glass coverslips using as mounting medium a 50% glicerol solution.

Images were acquired at 60X magnification with an FLUO VIEW 400-EVIDENT fluorescence microscope. For each condition, a range of 10 random fields per slide was analyzed.

EGFR activation and dimerization assay. $1x10^6$ BMDM were seeded in 60 mm dishes in complete medium to allow them to adhere overnight, serum-starved for 16 h and stimulated for 1 hr with 100 ng/ml of murine Egf (Cat. 2028-EG, Bio-techne) 250 ng/ml of murine recombinant Angiogenin (Cat. CSB-EP001703MO, Cusabio Technology LLC) on ice. Cells were then crosslinked with 3 mM BS3 (Sigma-Aldrich, Cat.no. 82436-77-9) for 20 min on ice and quenched with 250 mM glycine for 5 min at 4 °C. Pellets were lysed in ice-cold lysis buffer (140 mM NaCl, 10 mM EDTA, 10% glycerol, 1% NP-40, and 20 mM TRIS-HCl pH 8) supplemented with protease and phosphatase inhibitors, and supernatant was collected as whole cell extract (WCE) after centrifugation at 16,000 × g at +4 °C for 20 min. 50 µg proteins were resolved on a 6% SDS-polyacrylamide gel, transferred overnight onto a nitrocellulose membrane and incubated with anti-Egfr antibody (Santa Cruz Biotechnology, sc-373746).

Rho/Rac GTPase activity. The active GTPase pull-down assays were performed according to the manufacturer's instructions for the active RAC1 detection kit (Cat. No. 8815, Cell Signaling Technology) and active Rho Detection Kit (Cat. No. 8820, Cell Signal Technology), with samples treated with GTPγS (positive control) and GDP (negative control). The immunoprecipitated materials and total protein samples were then prepared for immunoblot analysis.

RNA extraction and real-time qPCR. RNA extraction was performed using PureLink RNA Mini Kit (Invitrogen) and quantified by using Nanodrop 8000. After DNase I treatment (Roche Diagnostics), 1 µg of RNA was reverse transcribed with High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems). Quantitative real-time PCR was carried out at 60°C using PowerUp SYBR Green Master Mix (Applied Biosystems) on a QuantStudio3 (Applied Biosystems). Primers were designed by using Primer3. Primer sequences are reported in Supplementary Table 1.

Chromatin Immunoprecipitation (ChIP).

For ChIP experiments, the RAW264.7 cell line was used instead of BMDM according to the 3R practices, given the requirement of high amount of cells. Briefly, $4x10^6$ RAW264.7 were fixed with 1% formaldehyde for 15 min, and fixation stopped by addition of 125 mM glycine for 5 min. Cells were lysed in Farnham lysis buffer (5 mM PIPES pH 8, 85 mM KCl, 0.5% NP-40) supplemented with protease inhibitors for 8 min. Nuclei were collected by centrifugation and lysed in nuclear lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8) supplemented with protease inhibitors for 20 min. Chromatin was sonicated with an ultrasonic bath (8 cycles, 30 sec ON, 30 sec OFF) to an average size of 200–600 bp, diluted with Dilution Buffer (10 mM Tris-HCl pH 8, 2 mM EDTA, 140 mM NaCl, 1% Triton X-100, 0.1% SDS) and incubated overnight with 20 µl dynabeads protein G and 3 µg of mouse anti-Smad2 (phospho-S465) (#ab216482, Abcam) or normal rabbit IgG (#2729, Cell Signaling Technologies). Immunocomplexes were washed with increasing salt concentrations, DNA eluted at 65°C with 1% SDS and recovered with PureLink RNA mini kit (Cat. 12183018A, Invitrogen). qPCR was carried out at 60°C using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) on a QuantStudio3 (Applied Biosystems). Promoter sequence of Tnf has been obtained at UCSC Genome Browser GRCm38/mm10 (chr17: 35203907-35206007). Primer sequences are reported in Supplementary Table 1.

Protein extraction, western blot and immunoprecipitation. For western blot, cells were lysed in cold RIPA buffer (Cat. ab156034, Abcam) supplemented with protease and phosphatase inhibitors (#5872, Cell Signaling Technologies) and centrifuged at 20000xg for 20min at 4°C. Protein quantification was performed using the Pierce BCA Protein Assay kit (Thermofisher Scientific). Equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and incubated for 1h in Every blot blocking buffer (Cat. No 12010020, Bio-Rad) at room temperature.

For protein immunoprecipitation experiments, 500 µg whole cell extract was incubated overnight with 50 µl of protein A/G PLUS-agarose beads (Santa Cruz Biotechnology, #sc-2003) and 2.5 µg of rabbit anti-TGFβR1 (Abcam, # ab31013), or normal rabbit IgG Isotype Control (Cell Signaling

Technologies, #3900). Proteins were eluted after boiling for 10 min at 95 °C in 2× Laemmli sample buffer (Bio-Rad, catalog no.: 1610747). Blotted membranes were developed by using Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore) and imaged with UVITEC Alliance Q9 Advanced (UVITEC Cambridge). Primary antibodies are reported in Supplementary Table 2.

Enzyme-linked immunosorbent assay (ELISA). ELISA assays were performed with mouse Angiogenin ELISA Kit (Novus Biologicals, Cat. No. NBP2-68208), mouse Tnfα ELISA Kit (R&D System Cat. no MTA00B), and mouse Tgfβ ELISA Kit (Novus Biologicals, Cat. no NBP1-92671). Conditioned media (CM) or plasma samples from mice were diluted before use following manufacturer's instructions. The optical density was determined with microplate reader iMark (Bio-Rad) at 540 nm. Human Angiogenin (Cat. No. DAN00) and human TNFα (Cat. No. DTA00D) ELISA kits from R&D System were used to assess ANG and TNFα levels in plasma samples from patients.

Immunohistochemistry. Immunohistochemistry (IHC) and hematoxylin and eosin (H&E) were performed on murine pancreatic sections of formalin-fixed paraffin-embedded specimens cut at 5 µm interval. After antigen retrieval with citrate buffer pH 6.0 (Leica Biosystems), tissues were blocked with 2.5% BSA blocking solution (Vector laboratories) for 1h and incubated with primary antibodies against CD68 or CD206 overnight at 4°C, or stained with H&E (cat. no H-3502, Vector Laboratories). Slides were developed using ImmPACT DAB substrate peroxidase HRP substrate (Vector Laboratories) following manufacturer's instructions. Primary antibodies are reported in Supplementary Table 2.

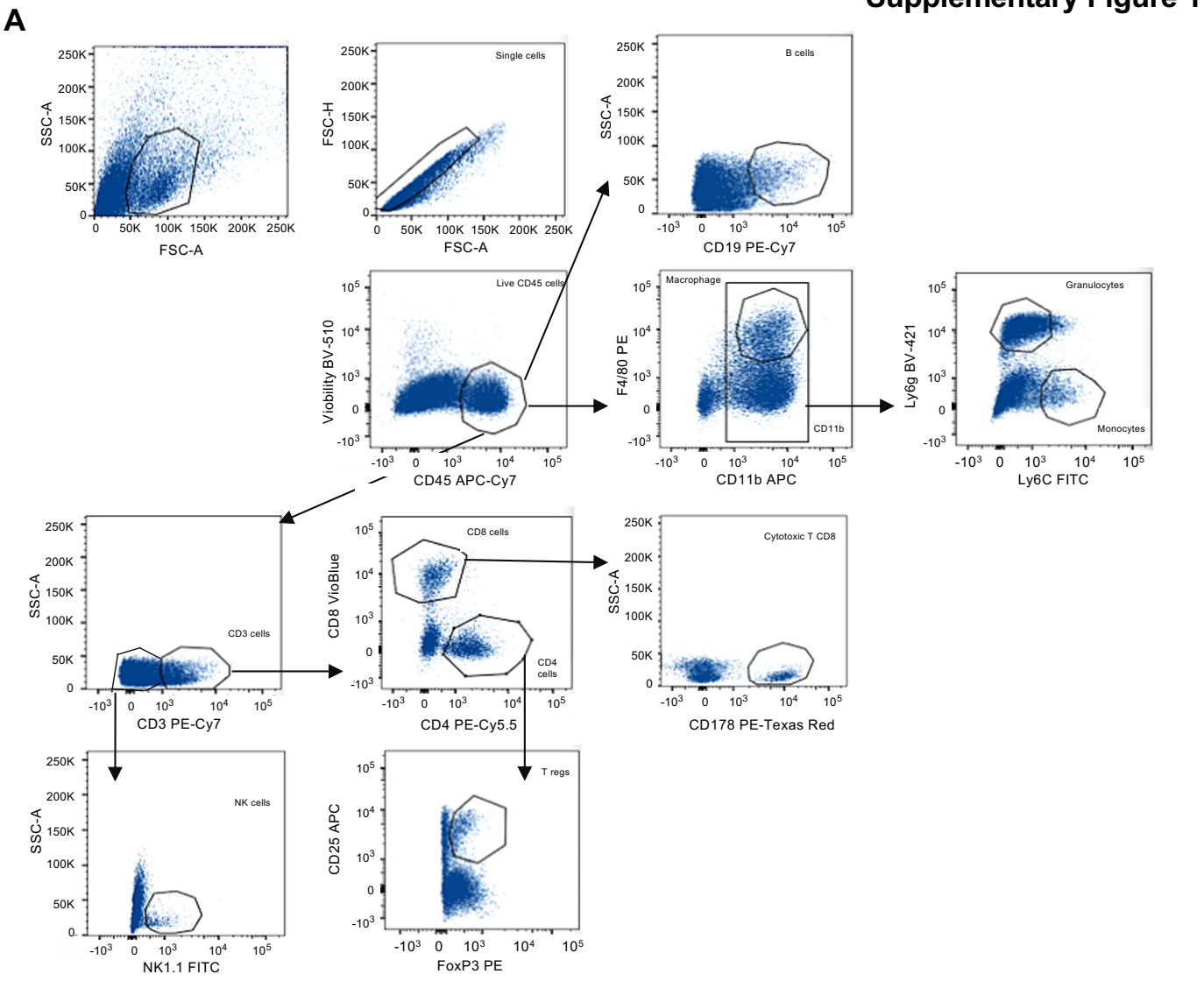
Flow cytometry. Tumors were dissociated using Tumor dissociation kit mouse (Cat. no 130-096-730, Miltenyi Biotec) according to manufacturer's instructions. Cells were collected, filtered by 70µm cell strainer and centrifuged for 5 min at 1000 rpm. Cell pellets (1×10^6) were resuspended in 100µL of PBS and incubated for 15 min at 4°C with a panel antibody cocktail of CD45 APC-Vio770 (REA737), CD11b APC (REA592), F4/80 PE (REA126), CD3 PE-Vio770 (REA641), CD8a VioBlue (53-6.7), CD178 PE-Vio 615 (REA1171), Ly6G VioBlue (REA526), Ly6C FITC (REA796), NK1.1

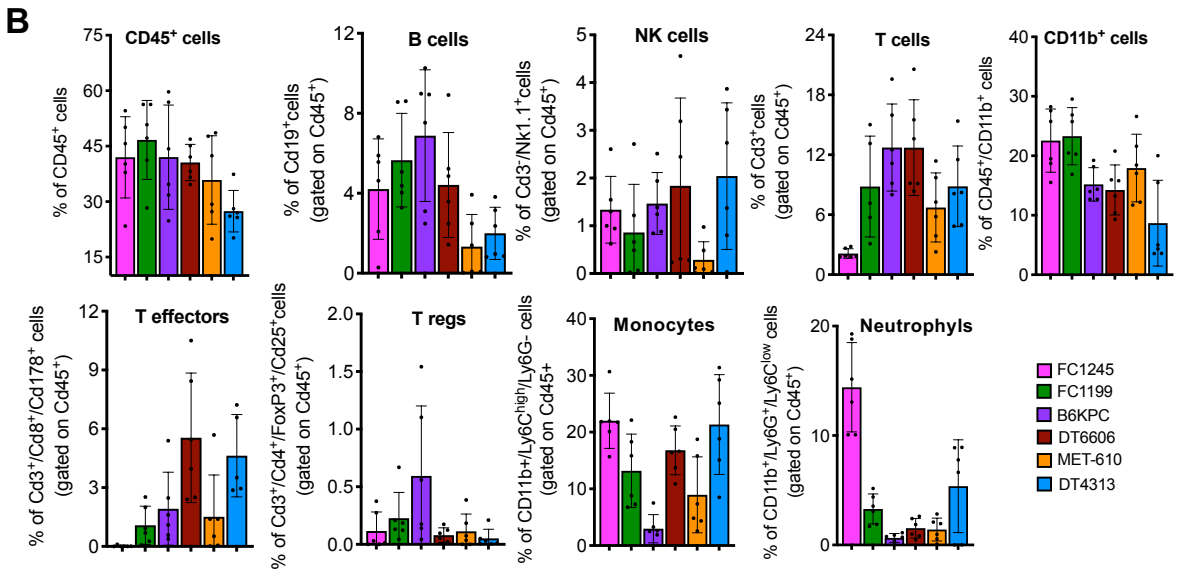
FITC (REA1162), all from Miltenyi Biotec. T reg staining was performed with MACS Inside Stain kit (Cat. 1 30-090-477) with the panel antibody cocktail of CD45 APC-Vio770 (REA737), CD4 PerCP-Vio700 (REA604), CD25 APC (REA568), FoxP3 PE (REA788), all from Miltenyi Biotec. Macrophage phenotype was evaluated using CD204 PE-Vio770 (C068C2) for M2 TAMs, and CD86 VioBright B515 (REA1190) for M1 TAMs, respectively. The Fixable die Viobility SYTOX Blue (Invitrogen) was used to gate for live cells. Acquisition was performed using Becton Dickinson LSR-Fortessa X-20, and samples were analyzed using FlowJo software (TreeStar Inc., Ashland, USA).

In vivo orthotopic transplantations. 5-6 weeks old C57BL/6J female with body weight ranging from 21 to 25g were purchased from Charles River Laboratories. Murine PDAC cells were resuspended in a PBS:Matrigel solution (1:1) at a concentration of $2.5X10^5$ cells per 40 µl/injection. On day 0, mice were anesthetized by exposure to isoflurane and injected orthotopically into the pancreas parenchyma. For in vivo treatments, mice bearing pancreatic cancer were randomly allocated into 8 groups (n=12) to receive galunisertib (100 mg/kg) via oral gavage twice/day, gemcitabine (75 mg/kg) intraperitoneally once/week, or their vehicles. For Kaplan-Meier survival analysis, 6 mice from each group were euthanized by cervical dislocation when tumors reached the ethical cutoff volume 1500 mm^3, and included in the survival curve. For flow-cytometry and ELISA, 6 mice from each group were euthanized by cervical dislocation at the end of 4-weeks treatment, peripheral blood was collected by retro-orbital sinus puncture, and tumors were collected for ex vivo analysis. Mice were maintained at the animal facility of the University of Verona, in a pathogen-free and temperature-controlled environment with 12h light and dark cycles, housed in plastic cages and fed ad libitum. Research involving animals was conducted according to the relevant guidelines and regulations and has been approved by the Italian Ministry of Health (authorization no. 299/2022-PR from Ministry of Health, prot. C46F4.29).

SUPPLEMENTARY FIGURES

Supplementary Figure 1





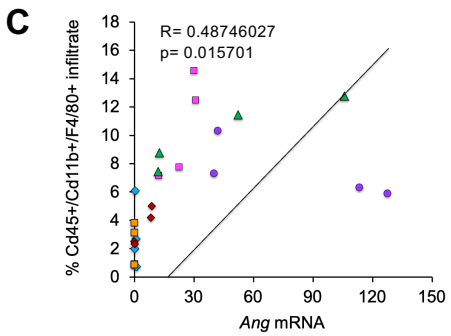


Figure S1: Immunophenotyping of orthotopic PDAC models. (A) Single-cell suspensions were gated for physical parameters, including forward scatter (FSC) for size, and side scatter (SSC) for granularity. Representative sample FACS plots of FC1245-bearing C57BL/6J mice are shown. **(B)** Flow cytometry analysis showing immune cell infiltration in FC1245, FC1199, B6KPC, MET610, DT6606 and DT4313 orthotopic tumors. Data are expressed as mean ± s.d. (n=6). **(C)** Pearson's correlation between Ang mRNA expression and the infiltration of TAMs in tumors from C57BL6/J PDAC models (n=24).

Supplementary Figure 2

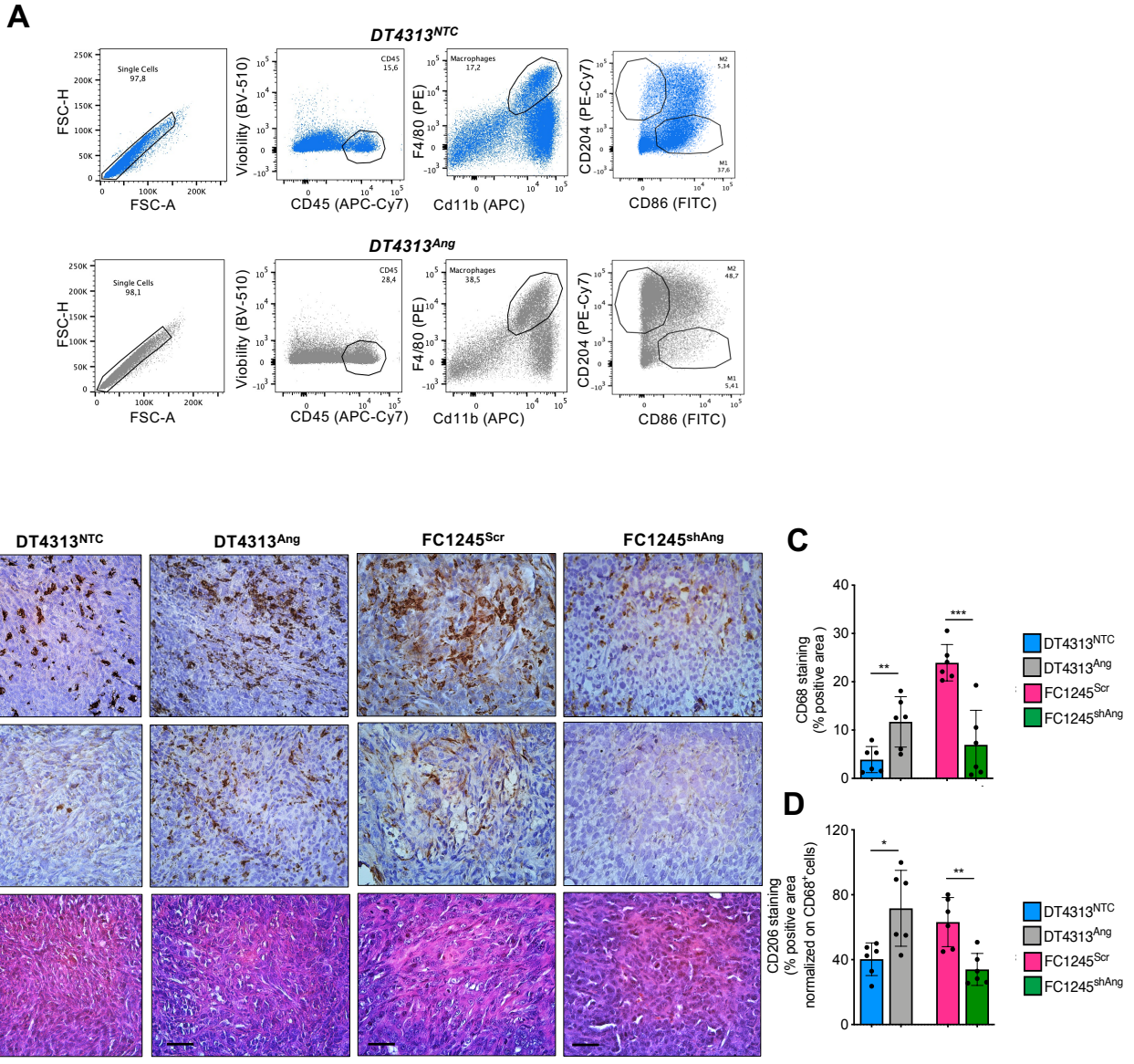


Figure S2: Tumor-derived Ang correlates with TAMs infiltrate in orthotopic PDAC models. **(A)** TAMs were gated based on $CD45^{+}/Cd11b^{+}/F4/80^{+}$ positivity, and evaluated for the expression of Cd86 and Cd204 (M1 TAMs: $Cd86^{+}/Cd204^{-}$; M2 TAMs: $Cd86^{-}/Cd204^{+}$). Sample FACS plots of $DT4313^{NTC}$ and $DT4313^{Ang}$-bearing C57BL/6J mice are shown. **(B-D)** Representative images of paraffin sections from $DT4313^{NTC}$, $DT4313^{Ang}$, $FC1245^{Scr}$ and $FC1245^{shAng}$ orthotopic tumors stained with Cd68 or Cd206 antibodies (B) and relative quantifications (C,D). Scale bar=60 μm. Data are expressed as mean ± s.d. (n=6). P values were calculated by two-tailed unpaired Student's t test. *p<0.05; **p<0.01; ***p<0.001.

Supplementary Figure 3

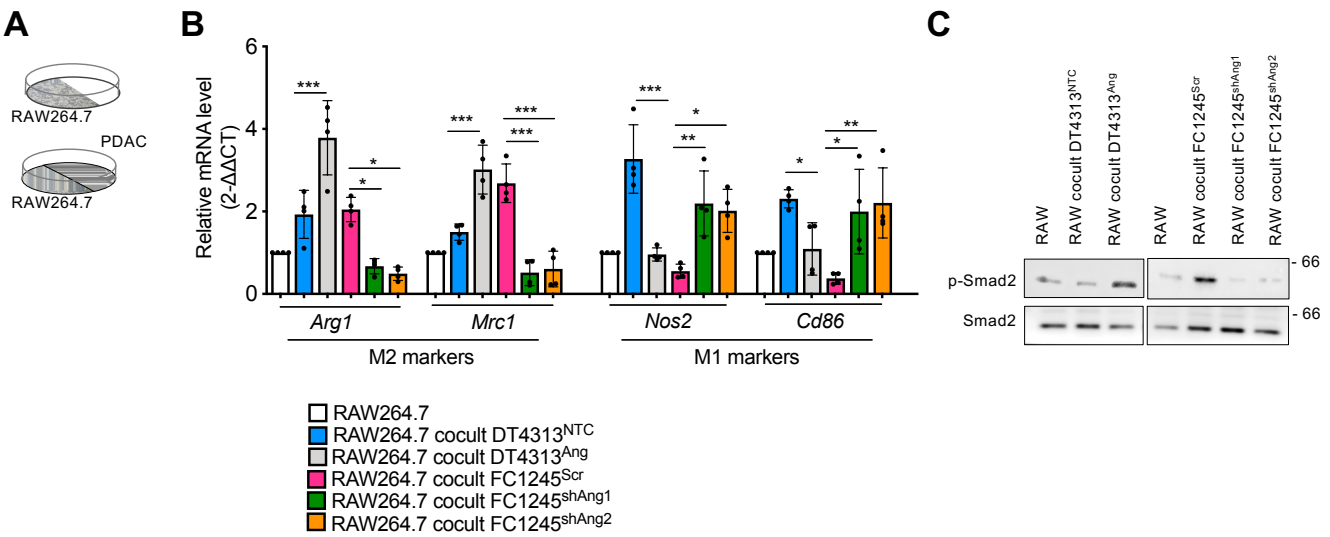


Figure S3: Ang induces M2-skewing of RAW264.7 macrophages in in vitro co-culture models. (A) Schematic representation of the co-culture technique used to culture RAW264.7 macrophages with PDAC cell lines. (**B**) qPCR analysis of M2 (Arg1, Mrc1) and M1 (Inos2, Cd86) markers in RAW264.7 as well as single cultures or co-cultured with DT4313 or FC1245 transduced as indicated. Data are shown as mean ± s.d (n=4). P values were calculated by ANOVA and Tukey's test. *p<0.05; **p<0.01; ***p<0.001. (**C**) Representative Western blot of pSmad2 and Smad2 in RAW264.7 as single cultures or co-cultured with DT4313 or FC1245 transduced as indicated.

Supplementary Figure 4

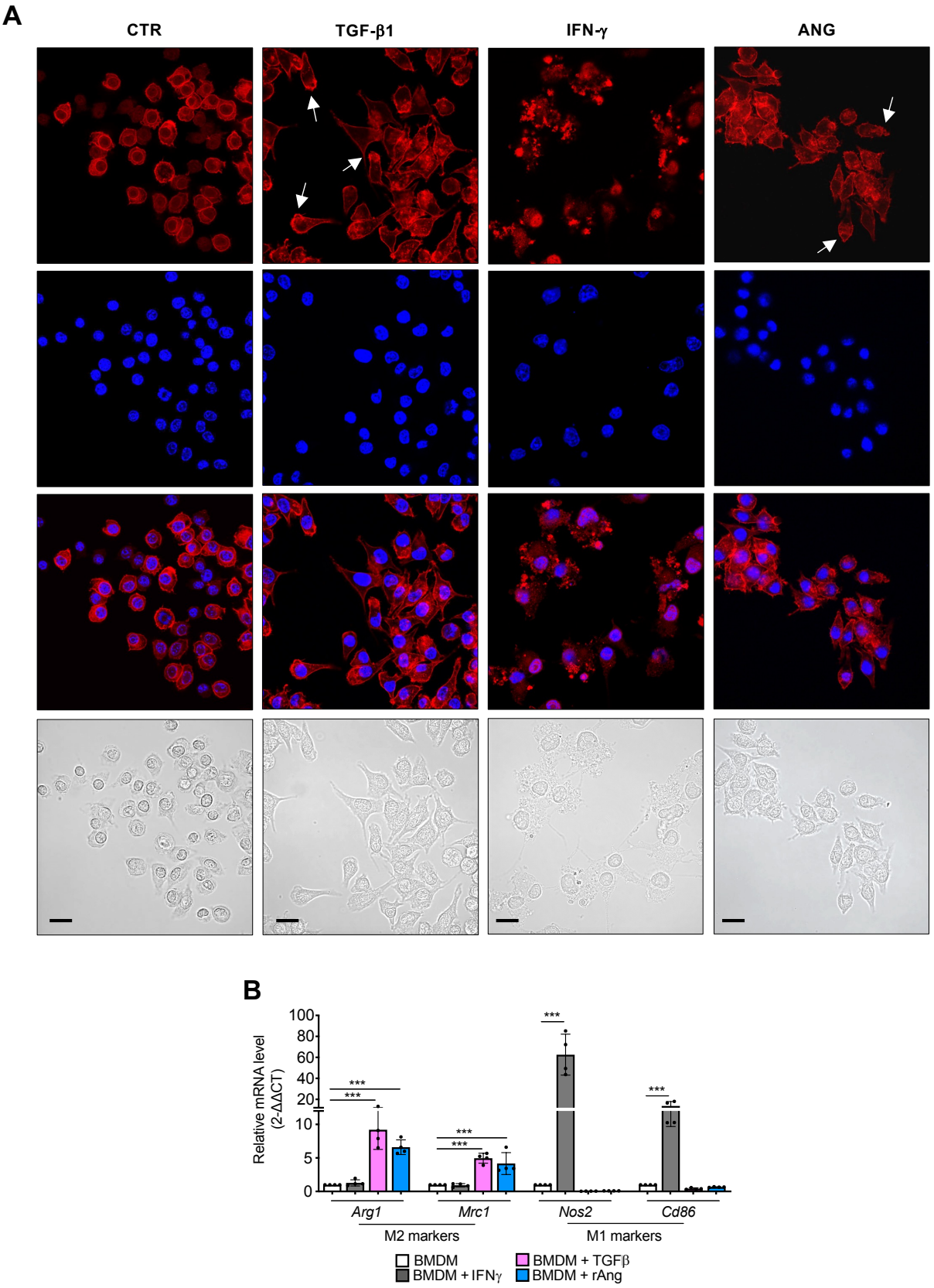
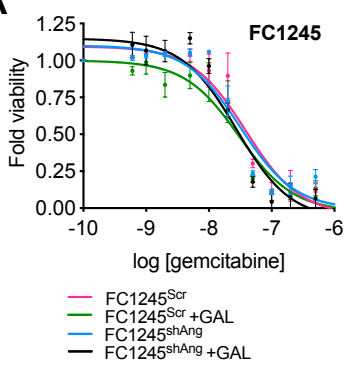
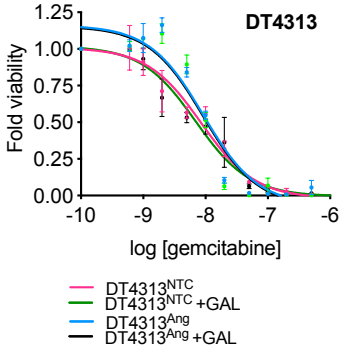


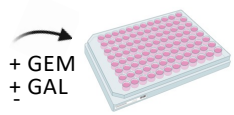
Figure S4: The M2 polarization state induced by Ang is associated with increased cellular elongation. (A) Representative immunofluorescence images of BMDMs cultured 48 hrs in RPMI medium and treated with Tgf-β1, Ifn-γ, rAng or their control vehicles (CTR). BMDMs were stained with Phalloidin and counterstained with DAPI. Scale bar = 20 μm. **(B)** qPCR analysis of M1 (*Nos2, Cd86*) and M2 (*Arg1, Mrc1*) markers in BMDM left unstimulated or stimulated with Tgfβ, Ifnγ or rAng. Data are shown as mean ± s.d (n=4). P values were calculated by ANOVA and Tukey's test. *p<0.001.

Supplementary Figure 5

A



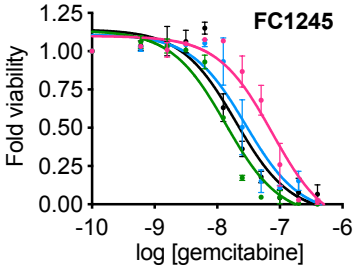


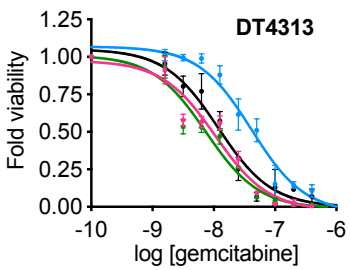


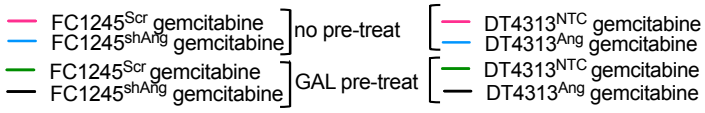
single cultures

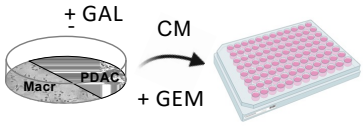
Treatments	EC50 GEM (nM)
$FC1245^{Scr}$	38.84 (95%CI: 25.28-59.67)
$FC1245^{Scr}$+ GAL	30.14 (95%CI: 19.28-47.11)
$FC1245^{shAng}$	31.83 (95%CI: 19.88-50.95)
$FC1245^{shAng}$+GAL	27.48 (95%CI: 18.00-41.95)
$DT4313^{NTC}$	8.52 (95%CI: 6.16-11.79)
$DT4313^{NTC}$+ GAL	6.93 (95%CI: 4.92-9.75)
$DT4313^{Ang}$	9.25 (95%CI: 6.09-14.06)
$DT4313^{Ang}$+ GAL	9.15 (95%CI: 5.98-13.99)

B





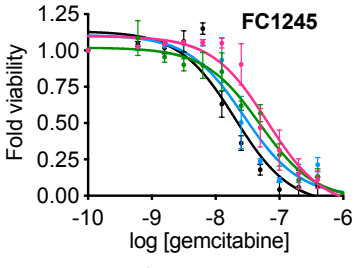


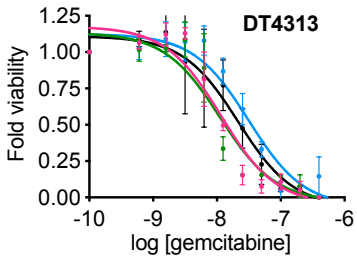


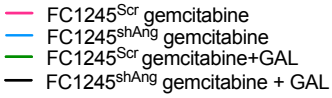
macrophages co-cult (CM) (pre-treated)

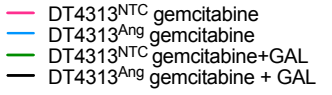
Treatments	EC50 GEM (nM)
$FC1245^{Scr}$	75.13 (95%CI: 51.1-110.5)
$FC1245^{Scr}$+ GAL	13.64 (95%CI: 9.64-19.29)
$FC1245^{shAng}$	28.29 (95%CI: 18.08-44.28)
$FC1245^{shAng}$+GAL	19.74 (95%CI: 12.65-30.79)
$DT4313^{NTC}$	10.55 (95%CI: 7.38-15.07)
$DT4313^{NTC}$+ GAL	7.43 (95%CI: 5.32-10.36)
$DT4313^{Ang}$	41.43 (95%CI: 30.21-56.81)
$DT4313^{Ang}$+ GAL	11.87 (95%CI: 8.27-17.04)

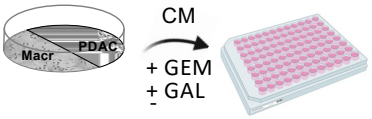
C









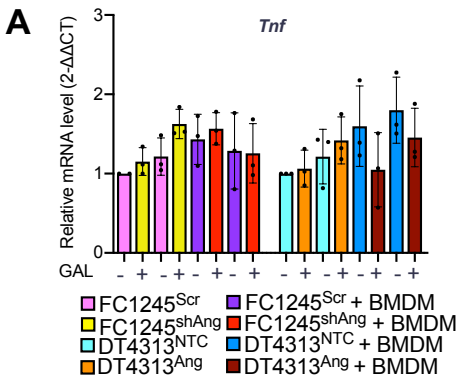


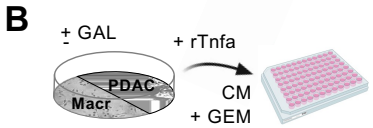
macrophages co-cult (CM)

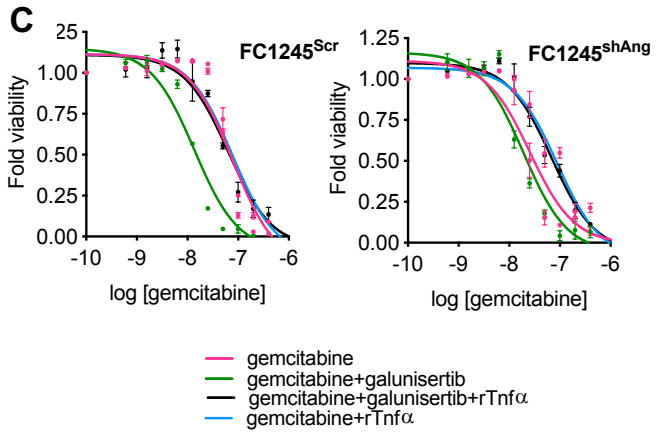
Treatments	EC50 GEM (nM)
$FC1245^{Scr}$	64.92 (95%CI: 43.85-96.14)
$FC1245^{Scr}$+ GAL	49.15 (95%CI: 35.88-67.35)
$FC1245^{shAng}$	27.42 (95%CI: 17.08-44.01)
$FC1245^{shAng}$+GAL	20.04 (95%CI: 12.87-31.22)
$DT4313^{NTC}$	11.61 (95%CI: 7.90-17.06)
$DT4313^{NTC}$+ GAL	11.16 (95%CI: 6.78-18.37)
$DT4313^{Ang}$	31.58 (95%CI: 20.53-48.59)
$DT4313^{Ang}$+ GAL	21.58 (95%CI: 12.60-36.95)

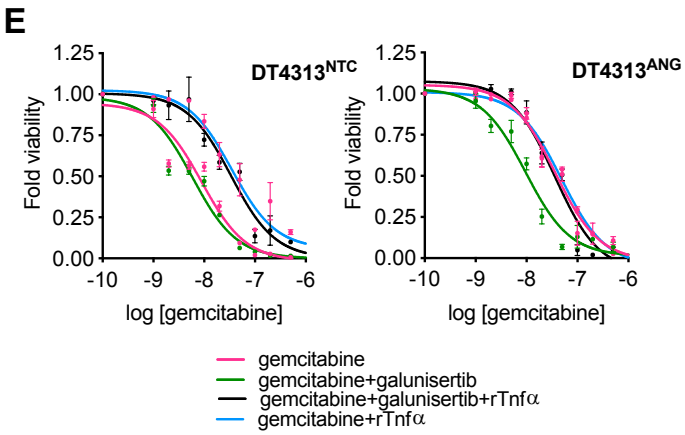
Figure S5: Inhibition of TGFβ in macrophages sensitizes Ang-high PDAC cells to gemcitabine-based chemotherapy. Dose-response curves in FC1245 or DT4313 after 72h of treatment with increasing doses of gemcitabine as single cultures (A), as co-cultured with BMDMs that were pre-treated with 2.5μM galunisertib (B) or as co-cultured with BMDMs and then treated with gemcitabine in combination with 2.5μM galunisertib. Tables indicates EC50 values and 95% CIs of gemcitabine. GEM: gemcitabine; GAL: galunisertib; CM: conditioned medium.

Supplementary Figure 6









D

Treatments	EC50 gemcitabine (nM)	
	NO rTnfα	+ rTnfα
FC1245^{Scr}	78.83 (95%CI: 48.04-129.4)	78.17 (95%CI: 53.8-113.6)
FC1245^{Scr}+ GAL	13.65 (95%CI: 9.60-19.41)	63.41 (95%CI: 41.72-96.37)
FC1245^{shAng}	25.66 (95%CI: 15.37-42.84)	79.52 (95%CI: 61.18-103.4)
FC1245^{shAng}+GAL	19.03 (95%CI: 12.31-29.43)	68.56 (95%CI: 50.44-93.17)

F

Treatments	EC50 gemcitabine (nM)	
	NO rTnfα	+ rTnfα
DT4313^{NTC}	8.97 (95%CI: 6.10-13.19)	35.04 (95%CI: 21.73-56.49)
DT4313^{NTC} + GAL	5.91 (95%CI: 4.07-8.57)	33.80 (95%CI: 20.66-55.30)
DT4313^{Ang}	36.79 (95%CI: 26.62-50.83)	48.45 (95%CI: 37.82-62.05)
DT4313^{Ang} +GAL	9.60 (95%CI: 6.68-13.80)	36.06 (95%CI: 24.90-55.21)

Figure S6: TAMs-derived Tnfα is the mediator of TGFβ signaling in sustaining chemoresistance of Ang-high PDAC cells. (A) qPCR of *Tnf* in FC1245^{Scr}, FC1245^{shAng}, DT4313^{NTC} or DT4313^{Ang} as single culture or in coculture with BMDMs and treated for 72hrs with 2.5μM galunisertib. Data are expressed as mean ± s.d. (n=3). P values were calculated using ANOVA and Tukey's test. **(B)** Schematic representation of the in vitro treatment schedule. **(C-F)** Dose-response curves of FC1245 (C,D) or DT4313 (E,F) transduced as indicated and co-cultured with BMDMs, after 72h of treatment with gemcitabine alone or in combination with 2.5μM galunisertib, in presence or absence of 20 ng/mL recombinant Tnfα (rTnfα). Tables indicates EC50 values and 95% CIs of gemcitabine. GAL: galunisertib; rTnfα: recombinant Tnfα.

Supplementary Figure 7

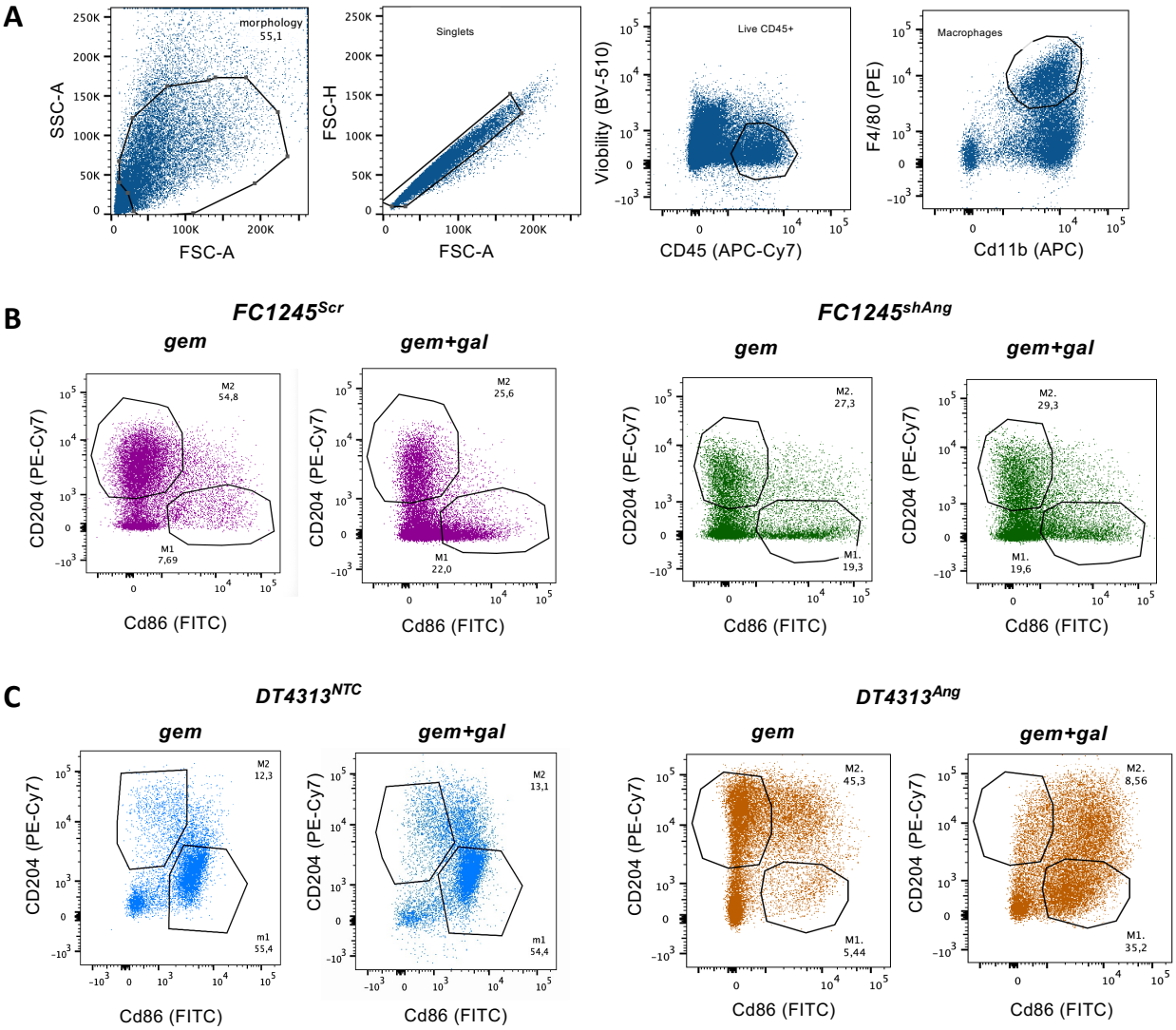


Figure S7: Gating strategy. Single-cell suspensions were gated for physical parameters, including forward scatter (FSC, size) and side scatter (SSC, granularity). TAMs were gated based on $CD45^{+}/Cd11b^{+}/F4/80^{+}$ positivity and evaluated for the expression of Cd86 and Cd204 (M1 TAMs: $Cd86^{+}/Cd204^{-}$; M2 TAMs: $Cd86^{-}/Cd204^{+}$). Sample FACS plots of $FC1245^{Scr}$, $FC1245^{shAng}$, $DT4313^{NTC}$ or $DT4313^{Ang}$ -bearing C57BL/6J mice treated with gemcitabine as single agent or in combination with galunisertib are shown. GEM: gemcitabine; GAL: galunisertib.

Supplementary Table 1. List of primers used in this study.

Primer	Sequence (5' to 3')
Ang Fwd	CCAGGCCCGTTGTTCTTG
Ang Rev	CCAGAGTGGGAGGGATCACA
Tgfb1 Fwd	TTACCTTGGTAACCGGCTGC
Tgfb1 Rev	AGCCCTGTATTCCGTCTCCT
Hprt Fwd	AGTCCCAGCGTCGTGATTAG
Hprt Rev	GCCTCCCATCTCCTTCATGA
Arg1 Fwd	CTGAGCTTTGATGTCGACGG
Arg1 Rev	TCCTCTGCTGTCTTCCCAAG
iNos2 Fwd	CCCCGCTACTACTCCATCAG
iNos2 Rev	CCACTGACACTTCGCACAAA
Tnf Fwd	GTGCCTATGTCTCAGCCTCT
Tnf Rev	CTGATGAGAGGGAGGCCATT
Mrc1 Fwd	ATGGGCAACATCGAGCAGAA
Mrc1 Rev	AAACCAATGCAACCCAGTGC
Cd86 Fwd	CCGGATGGTGTGTGGCATAT
Cd86 Rev	TGAGCAGCATCACAAGGAGG
Tnf prom A Fwd	TCACATCCCCACAGTCTCCA
Tnf prom A Rev	TCCCATGTCTGTCCCTCCTT
Tnf prom B Fwd	GGGTGGAGAGAGATGAGGGT
Tnf prom B Rev	CCAATCCGTATGACTCCCCG

Supplementary Table 2. List of antibodies used in this study.

Antibody	Source	Cat. No.	Dilution
Ang	Invitrogen	14017.7	1:1000 (WB)
Egfr	Abcepta	AM7628b	1:1000 (WB)
phospho-Egfr Tyr 1068 (D7A5)	Cell Signaling Technology	3777	1:1000 (WB)
Plexin B2	Santa Cruz Technologies	sc-373930	1:500 (WB)
phospho-Tyr-1000	Cell Signaling Technology	8954	1:1000 (WB)
TgfβRI	Abcam	ab31013	2.5 μg
Nf-kB (C22B4)	Cell Signaling Technology	4764	1:1000 (WB)
phosho-NF-kB Ser 536 (93H1)	Cell Signaling Technology	93H1	1:1000 (WB)
phospho-Smad2 Ser 465/467 (138D4)	Cell Signaling Technology	3108	1:1000 (WB) 1:200 (IHC)
Smad2	Cell Signaling Technology	3103	1:1000
Tgfβ1	RayBiotech	102-11520	1:1000 (WB)
phospho-Erk1/2 Thr 202/Tyr 204 (20G11)	Cell Signaling Technology	4376	1:1000 (WB)
Erk1/2 (L34F12)	Cell Signaling Technology	sc-13119	1:1000 (WB)
phospho-Stat3 Tyr 705	Cell Signaling Technology	9131	1:1000 (WB)
Stat3 (124H6)	Cell Signaling Technology	9139	1:1000 (WB)
Actin	Santa Cruz Technologies	sc-47778	1:5000 (WB)
Hsp90	Santa Cruz Technologies	sc-13119	1:10000 (WB)
Gapdh	Thermo Fisher Scientific	G9545	1:50000 (WB)
RhoA	Cell Signaling Technology	2117	1:1000 (WB)
Rac1	Cell Signaling Technology	8631	1:500 (WB)
CD68	R&D system	MAB101141	1:100 (IHC)
CD206	Abcam	ab64693	1:1000 (IHC)

WB: Western blotting; IHC: Immunohistochemistry.