T Cell-Mediated Antitumor Immunity Cooperatively Induced By TGF β R1 Antagonism and Gemcitabine Counteracts Reformation of the Stromal Barrier in Pancreatic Cancer

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ABSTRACT

The desmoplastic stroma of pancreatic cancers forms a physical barrier that impedes intratumoral drug delivery. Attempts to modulate the desmoplastic stroma to increase delivery of administered chemotherapy have not shown positive clinical results thus far, and preclinical reports in which chemotherapeutic drugs were coadministered with antistromal therapies did not universally demonstrate increased genotoxicity despite increased intratumoral drug levels. In this study, we tested whether TGF β antagonism can break the stromal barrier, enhance perfusion and tumoral drug delivery, and interrogated cellular and molecular mechanisms by which the tumor prevents synergism with coadministered gemcitabine. TGF β inhibition in genetically engineered murine models (GEMM) of pancreas cancer enhanced tumoral perfusion and increased intratumoral gemcitabine levels. However, tumors rapidly adapted to TGF β -dependent stromal modulation, and intratumoral perfusion returned to pre-treatment levels upon extended TGF β inhibition.

Perfusion was governed by the phenotypic identity and distribution of cancer-associated fibroblasts (CAF) with the myelofibroblastic phenotype (myCAFs), and myCAFs which harbored unique genomic signatures rapidly escaped the restricting effects of TGF β inhibition. Despite the reformation of the stromal barrier and reversal of initially increased intratumoral exposure levels, TGF β inhibition in cooperation with gemcitabine effectively suppressed tumor growth via cooperative reprogramming of T regulatory cells and stimulation of CD8 T cell-mediated antitumor activity. The antitumor activity was further improved by the addition of anti-PD-L1 immune checkpoint blockade to offset adaptive PD-L1 upregulation induced by TGF β inhibition. These findings support the development of combined antistroma anticancer therapies capable of impacting the tumor beyond the disruption of the desmoplastic stroma as a physical barrier to improve drug delivery.

Introduction

Pancreatic cancer is a fatal disease which remains persistently refractory to current treatment options (1, 2). 5-year survival rates of 5% to 8% have not substantially changed over the last three decades as survival gains of applied systemic chemotherapy treatment combinations continue to be measured in months with only sporadic, and largely anecdotal, reports of cures (3–5). One of the unique histopath-

ologic hallmarks of pancreatic cancer is the presence of an abundant desmoplastic stroma characterized by high interstitial pressures and increased collagen content leading to poor vascularization and restricted accessibility of cancer therapeutics (6, 7).

The concept of breaking the stromal barrier to improve anticancer therapy initially focused on increasing delivery of cytotoxic chemotherapy to tumor cells (8, 9). However, stromal modulation via inhibition of sonic hedgehog (SHH) signaling after initial promising

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preclinical reports failed to show benefit in patients with pancreatic cancer (10, 11). Further postclinical studies then recognized a potentially harmful procarcinogenic effect of stromal SHH modulation capable of attenuating tumor suppressive mechanisms within the tumor microenvironment (TME; 12). Similarly, clinical reports on the use of pegylated human hyaluronidase (PEGPH₂₀), which in preclinical studies via enzymatic degradation of the macromolecule hyaluronan effectively reduced stromal pressures and increased tumoral perfusion, have been disappointing when PEGPH₂₀ was coadministered with chemotherapeutics (9, 13). That stromal modulation and coadministered anticancer therapy can also elicit effective, synergistic antitumor responses independent from breaking the physical barrier of the TME and improved intratumoral accessibility of anticancer therapeutics, was recently shown in genetically engineered murine models (GEMM; refs. 14, 15). After uncoupling intratumoral gemcitabine concentrations from stromal depletion, increased intratumoral gemcitabine concentrations were not universally associated with increased apoptosis level indicating that (i) increasing intratumoral gemcitabine concentrations alone are not associated with improved anticancer response, and (ii) there are additional cooperative, yet-to-be-discerned mechanisms which mediate tumor growth arrest (14). Stromal modulators might thus be able to govern tumor control independent of coadministered chemotherapy as poor tumoral perfusion and associated regional and global hypoxia have been linked to loss of tissue homeostasis and tumor immune surveillance (16), involving impaired T-cell functions (17), a shift of tumor-associated macrophages towards the immune suppressive M2-like phenotype (18), or the upregulation of inhibitory immune checkpoints (19). Intriguingly, it has been suggested that response to checkpoint therapy can be improved by increasing perfusion (16). Thus, future questions for effective combined anti-stroma/antitumor responses will need to address mechanisms of resistance to stromal modulation as well as an improved understanding of cellular elements involved in regulating tumor tissue perfusion.

To better understand the role of the physical barrier of the stroma including its involved cellular elements, and to examine novel, cooperative mechanisms of action of a dual antistroma anticancer approach beyond increased drug delivery, we evaluated a small-molecule inhibitor targeting TGF β signaling administered at low dose in combination with gemcitabine in autochthonous murine models of pancreas cancer. We show that low dose T β R-I inhibition had minimal effects on epithelial cancer cell differentiation but effectively increased tumoral perfusion and drug delivery. Despite rapid adaptation of the stromal barrier due to treatment-induced resistance in the myelofibroblastic subset of cancer-associated fibroblasts (myCAF), TGF β blockade in combination with gemcitabine cooperatively altered the immune landscape of tumor tissue, including reprogramming of T regulatory cells and activation of cytotoxic CD8⁺ T cells that led to improved disease outcomes. These outcomes can be further enhanced by the addition of PD-L1 checkpoint blockade to employ combination therapy to target an adaptive mechanism of resistance.

Methods

Animal strains

Colonies of transgenic mice were established according to protocols and policies of NIHs Institutional Animal Care and Use Committees. Mice with individual alleles for Pdx-1-cre, LSL-Kras^{G12D/+}, and Ink4a (p16)/Arf(p19)^{flox/flox} or LSL-Trp53^{R172H/+} were crossed to create animals with the triple genotype Pdx-1-cre;LSL-Kras^{G12D/+};Ink4a (p16)/Arf(p19)^{flox/flox} (KP16) or Pdx-1-cre;LSL-Kras^{G12D/+};LSL-Trp53^{R172H/+} (KPC; refs. 20, 21).

Animal imaging

Mice with the KP16 genotypes were examined by weekly abdominal ultrasound imaging starting at six weeks of life, or for the KPC model when abdominal palpation revealed an abdominal mass ≥ 0.5 cm. Ultrasound imaging was performed using a 40 MHz transducer and a Vevo700 ultrasound machine (Visualsonics). Dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) was performed using a 7T Avance MRI scanner (Bruker-Biospin). Mice were anesthetized with isoflurane (Baxter) and placed on a homemade cradle in a 35-mm birdcage MRI probe. A FLASH dynamic MRI scan was completed before and 5 minutes after the administration of a 100 μ L bolus of intravenous Magnevist contrast (Berlex Laboratories). The scan parameters were FOV = 3 cm, slice thickness = 1 mm, flip angle = 60 degrees, and repetition time = 90 msecs.

Treatment protocols

In all animals, drug administration started after ultrasound confirmed a pancreatic tumor measuring ≥ 4 –6 mm in largest linear dimension or 200–250 mm³ in total volume for KP16 and ≥ 5 –10 mm or 250–500 mm³ for KPC tumors. Mice were randomized to respective treatment arms. LY364947 (Sigma-Aldrich) was diluted in 200 μ L PBS and injected intraperitoneally (i.p.) at 1 mg/kg body weight as described previously (22). Gemcitabine (Sigma-Aldrich, catalog no. G6423) was diluted in 100 μ L normal saline and injected i.p. every 4 days at 50 mg/kg. Anti-PD-L1 (BioLegend, catalog no 124329) was administered twice a week at 150 μ g via i.p. injection, for CD8 T-cell depletion, two doses of 100 μ g anti-mCD8 (Bioxcell, catalog no BE0061) or rat isotype control IgG₁ (Bioxcell, catalog no BE0090) per mouse on day 1 and 5 were administered.

Perfusion assays

Immunofluorescent FITC-dextran perfusion was performed at the end of 2-, 7-, and 14- day treatment courses. Six hours prior to euthanasia, animals received 200 μ L of 10 mg/mL fluorescein isothiocyanate-dextran (Thermo Scientific, catalog no. D7137) via tail vein injection. Following euthanasia, tumors and spleens were fixed in formalin for 48 hours, and embedded in paraffin. Four-micron-thick sections were imaged on a Zeiss 710 confocal microscope. An automating tiling algorithm to image the entire section of tumor and spleen tissues was employed. Multiple rows of individual scans (up to several hundred) were digitally combined into a single image. The Zen 2009 software (Zeiss) was used to complete post-acquisition image processing. Assessment of functional vasculature via detection of intravenously administered lectin within CD31⁺ vascular structures was done as described previously (8).

Gemcitabine perfusion studies were performed at the completion of the 2-day and 14-day experiments. Animals received 50 mg/kg gemcitabine i.p. 2 hours prior to euthanasia, and harvested tumors were measured for intratumoral gemcitabine metabolites, as described previously (23). Whole tissue concentrations of gemcitabine (2',2'-difluoro-2'-deoxycytidine, dFdC), its metabolite 2',2'-difluoro-2'-deoxyuridine (dFdU), and the active metabolite gemcitabine 5'-triphosphate (2',2'-difluorodeoxycytidine-5'-triphosphate, dFdCTP) were measured using a LC/MS-MS system. In brief, whole tumors were flash frozen in liquid nitrogen and homogenized in ice-cold acetonitrile (50% v/v) containing 25 μ g/mL tetrahydrouridine (Promega) using a Precellys 24 homogeniser (Stretton Scientific Ltd.) to make a final concentration of 50 mg tissue per mL. Fifty microliters of homogenate was transferred to a clean tube prespiked with 200 μ L of ice-cold acetonitrile containing 50 ng/mL stable labeled internal standards. After centrifugation at 20,000 $\times$ g for 25 minutes, the

supernatant was evaporated to dryness. The residue was reconstituted in 100 μ L water and injected into the mass spectrometer. LC/MS-MS was performed on a triple-stage quadrupole Vantage mass spectrometer (Thermo Scientific) fitted with a heated electrospray ionisation (HESI-II) probe operated in positive and negative mode at a spray voltage of 2.5 kV and capillary temperature of 250°C.

Histology

All organs were fixed in 10% neutral buffered formalin for 48 hours prior to paraffin-embedding and sectioning. All IHC staining was completed on the Leica Biosystems BOND MAX platform (Buffalo Grove, IL), antibodies used for chromogenic IHC staining and tissue immunofluorescence are listed in Supplementary Table S1.

IHC images were acquired using the Aperio AT2 Whole Slide Digital Scanner (Leica Biosystems) at 20 $\times$ magnification. Quantitative analysis was performed by Aperio Image Analysis Software (Leica Biosystems) employing membrane (E-cadherin, CK19), nuclear (Ki67), and positive pixel count algorithms (collagen, smooth muscle actin).

For phospho-SMAD2/SMA immunofluorescence costaining, 5- μ m-thick sections were deparaffinized, followed by 10 minutes of heat-induced epitope retrieval in citrate (phospho-SMAD2/SMA) or EDTA buffer (phospho-STAT3/SMA double staining). Primary antibodies were incubated for 60 minutes and secondary antibodies for 30 minutes with UltraCruz background blocking performed one hour prior. Tissues were manually counterstained with DAPI combined with mounting medium. Fluorescent images were acquired using the Aperio FL Multi-channel Fluorescence Slide Scanner (Leica Biosystems) at 20 $\times$ magnification, and quantitative analysis was performed by HALO Image Analysis (Indica Labs) utilizing the High-Plex FL v3.0.3 Classifier Algorithm.

Flow cytometry analysis

Harvested pancreatic tumors were rinsed with PBS, minced with a scalpel, and digested using 1 mg/mL of type IV collagenase (Sigma-Aldrich) and a Gentle Macs Agitator (Miltenyi Biotec). Tumor digests were passed through a 70- μ m filter, washed in PBS, and stained for flow cytometry analysis with antibodies listed in Supplementary Table S1. Stained cells were washed with FACS buffer prior to sample acquisition with the BD LSRFortessa SORP I flow cytometer (BD Biosciences). Flow cytometry data was analyzed using FlowJo software (TreeStar).

scRNA-seq library preparation

Single-cell suspensions prepared after dissociation of normal, uninvolved pancreas or pancreatic tumor tissues from C57B/L wild-type or tumor-containing KPC mice were washed with PBS + 0.04% BSA, resuspended in 1 mL of the same buffer and counted. On the basis of the viability, approximately 9,000–15,000 cells per sample were used to generate 10X Genomics scRNAseq libraries as per the manufacturer guidelines (10X Genomics). Briefly, cells, along with the barcode beads (5'v1, 3'v2 or 3'v3) and RT reagents were loaded onto GEM generation chips (on separate channels for cells from separate animals) and GEMs (Gel Beads-in-emulsion) were generated using the 10X Genomics Chromium Controller. The barcoded cDNA generated after the Reverse Transcription step was purified, amplified for 12–15 cycles, cleaned up using SPRIselect beads (Beckman), and then run on Bioanalyzer (Agilent Technologies) to determine cDNA concentration. 3' or 5' mRNA-seq gene expression sequencing libraries were prepared from the cDNA as recommended by their respective 10X Genomics user

guides with appropriate modifications to the PCR cycles based on calculated cDNA concentration.

The libraries were sequenced on multiple NextSeq500 asymmetric paired-end runs with read length of 8 bp for the sample index read, and the following for Read 1 and Read 2: 28 bp Read 1 and 55 bp Read 2 for 3' v3 libraries; and 26 bp Read 1 and 57 bp Read 2 for 3' v2 and 5' v1 libraries (Illumina).

scRNA-seq data processing

Cell Ranger (version 3.1.0) was applied to all tumor and normal pancreas samples to perform sequencing depth normalization. Seurat (version 2.3.4) was used for initial data analysis. Genes detected in fewer than 3 cells and cells containing less than 200 genes were removed from the data sets prior to further analysis. In addition, cells with higher mitochondrial expression greater than 5% total determined by the Unique Molecular Identifier (UMI) tool were filtered out. After removing unwanted cells, data was normalized by a global-scaling normalization method logNormalize from Seurat. Scran and Seurat were used for clustering. Optimal numbers of Principal Components (PCs) were calculated in URD (<https://github.com/farrellja/URD>) and clustering results were evaluated with silhouette plots. Uniform manifold approximation (UMAP) and *t*-stochastic neighbor embedding (tSNE) were used for data visualization in two-dimensional spaces (24).

For cell type identification, we used SingleR (version 1) and applied the pre-labeled "Single-Cell Analysis of Pancreatic Ductal Adenocarcinoma" data set from Elyada and colleagues as reference (25). SingleR was trained to label new cells from our dataset based on similarity to the reference marker genes. Tumor-associated fibroblasts (CAF) including myCAF, iCAF, and apCAF subpopulations were identified using DigitalCellSorter (<https://github.com/sdomanskyi/DigitalCellSorter>) with marker genes previously identified in the KPC Tumor data set from the study of Elyada and colleagues. For the identifying of marker genes for each cell type the FindMarkers function of the Seurat package was used employing pair-wise comparisons of two selected cell type groups, or using one selected cell type subpopulation against all cells. The logfc.threshold = 0.25 was used. The Gene Set Enrichment Analysis (GSEA) was conducted with R package EnrichR (<https://rdrr.io/cran/enrichR/>).

scRNA-seq data files of both KPC tumors and normal pancreas have been deposited under NCBI GEO series GSE180859 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE180859>).

CNV prediction and validation

CNVs were inferred from scRNA-seq data using inferCNV R package (v1.0.4) (<https://github.com/broadinstitute/inferCNV>). For the identification of CNV changes in CAFs in KPC tumors, CAFs were compared with normal pancreas fibroblasts. The normal ductal cells were compared with tumor ductal cells to profile CNV changes in tumor cells. Identified CNVs were validated against a previously published array-based comparative genomic hybridization (array CGH) dataset of KPC tumors (26). CNVs were validated against reported CNVs in the previous study of Niknafs and colleagues. Bedtools version 2.27.1 was used to probe for overlap of CNVs with CNVs inferred from our scRNA-seq dataset. For the gene set functional classification of the validated CNVs DAVID version 6.8 (<https://david.ncicrf.gov/summary.jsp>) was used.

ELISpot assays and tumoral collagen content

CD8a⁺ T cells were purified from single-cell suspensions of whole digested tumors or spleens by EasySep Mouse CD8a Positive Selection

Kit II (catalog no. 18953, StemCell). A total of 1×10^5 KP16 cancer cells were cocultured for 20 hours with 4×10^4 isolated CD8 $^+$ T cells isolated in flat-bottom 96-well PVDF-membrane microtiter plates (catalog no. MAIPSWU10, EMD Millipore). Visualization of immobilized cytokine as ImmunoSpots was carried out according to the manufacturer's instructions (catalog no. 3321-2A, Mabtech), ELISpots were read and quantified in an ImmunoSpot S6 universal analyzer. T regulatory cells were isolated using the EasySep Mouse CD4 $^+$ CD25 $^+$ Regulatory T Cell Isolation Kit II (catalog no. 18783, StemCell). A total of 4×10^4 CD4 $^+$ CD25 $^+$ cells were added to 1×10^5 KP16 cancer cells and 4×10^4 splenic CD8 $^+$ T cells isolated from nontreated, tumor-bearing animals. To measure tumoral collagen content ($\mu\text{g}/\text{mg}$ wet tumor tissue), the Total Collagen Assay Kit (catalog no. ab222942, Abcam) was used following the manufacturer's instructions.

qRT-PCR

Gene expression levels in isolated CD8 and T regulatory cells was measured after RNA isolation using the RNeasy Mini Kit (catalog no. 74104, Qiagen), first strand cDNA synthesis (SuperScript III First-Strand, catalog no. 11752050, Thermo Fisher Scientific), and PCR amplification with individual primer master mix listed in Supplementary Table S1. qRT-PCR reactions were carried out and read in a Bio-Rad CFX96 cyclor.

Global gene expression profiling-TCGA data analysis

Genomic data from TCGA project are available from the National Cancer Institute's Genomic Data Commons (<https://gdc.cancer.gov/>). Gene-level gene expression datasets from RNA-seq analyses of tumors ($N = 9,452$) were downloaded from Cbioportal (<https://www.cbioportal.org>) and mean gene expression levels between individual groups were compared using GraphPad Prism (Version 7.01; 27).

Statistical analysis

Continuous data, including mean pancreatic tumor/spleen dextran intensity, MRI tumor pancreatic tumor/skeletal muscle mean intensity, gemcitabine metabolite concentration, tumor volumes, and immune cell population percentages were compared by paired Student *t* test and for multiple comparisons by one-way ANOVA with a *post hoc* Tukey test using GraphPad Prism (Version 7.01). Error bars indicate standard error of the means (SEM) unless otherwise indicated. Calculated *P* values were given by number and asterisk(s). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Results

Stromal adaptation to extended TGFβ antagonism reduces tumoral perfusion and drug delivery

The TGFβ receptor I (TβR-I) inhibitor LY364947 (Supplementary Fig. S1A) has been previously shown to remodel the stroma and increase delivery of cancer-targeting agents when administered at low dose to xenotransplantation models of desmoplastic solid organ cancers (22). We first investigated whether TβR-I blockade in autochthonous, Ras-driven KP16 and KPC tumors is equally able to reduce the stromal barrier and improve drug delivery. TβR-I blockade with LY364947 administered at the low dose of 1 mg/kg rapidly, within 48 hours from treatment start, reduced both collagen I content and smooth muscle actin-positive (SMA $^+$) cell fractions (Fig. 1A; Supplementary Fig. S1B). No impact on tumoral cell proliferation was observed when enumerating Ki67-positive cells. Surprisingly, upon continuation of LY364947 treatment, the initially induced

changes in reduced collagen and SMA expression patterns largely disappeared at the end of 14-day treatment protocol (Fig. 1A).

To examine whether tumoral perfusion follows these stromal changes, we investigated global perfusion in these tumors next. The intravenous administration of 2 MDa dextran molecules enables reliable assessment of such vascular and perfusion properties (28). At baseline, tumor perfusion measured about approximately 20%–25% of perfusion observed in the spleen dissected from the same animal. Administration of LY364947 markedly improved the dextran perfusion after 2 days which was also observed by perfusion weight DCE-MRI imaging where pretreatment with the TβR-I inhibitor increased gadolinium uptake compared with control (Fig. 1B and C). However, and in line with the lack of reduced stromal collagen I deposition and SMA $^+$ cell suppression upon extended treatment, the observed increase in perfusion at the end of treatment day 2 was only transient and perfusion levels returned to baseline when extending treatment to 7 or 14 days (Fig. 1B). Similar adaptation of intratumoral perfusion to prolonged TβR-I inhibition was observed in KPC tumors using an alternative perfusion assay. Following labelling of erythrocytes with lectin, perfusion levels were quantified as the ratio of CD31 $^+$ vessels containing labeled erythrocytes ("perfused") to nonperfused "collapsed" vessels lacking endovascular lectin labeling (Fig. 1D). Corroborating the findings obtained in the dextran perfusion assays, we found that perfusion initially increased approximately 2-fold after 2 days of treatment with LY364947, whereas no differences in intratumoral perfusion levels were observed after prolonged treatment (Fig. 1D).

Next, we examined whether intratumoral gemcitabine delivery followed above changes using a previously validated high-performance LC/MS-MS platform (23). Upon 48-hour pretreatment with the TβR-I inhibitor, KP16 tumors showed a marked increase in both active, intracellular 2',2'-difluorodeoxycytidine-5'-triphosphate (dFdCTP) as well as inactive 2',2'-difluorodeoxyuridine (dFdU) metabolite levels, whereas after prolonged stromal modulation, in line with the reversal of stromal alterations and return of perfusion levels to baseline levels, no increase in dFdCTP level was detected and dFdU concentration was only slightly increased (Fig. 1E). Of note, treatment with gemcitabine did not negatively affect subsequent gemcitabine drug delivery as intratumoral gemcitabine concentration did not differ between vehicle or TβR-I inhibitor-treated tumors for 14 days prior to receiving one dose of gemcitabine before tumor harvest, or tumors that continuously received gemcitabine or gemcitabine in combination with the TβR-I inhibitor (Supplementary Fig. S1C). Thus, stromal characteristics governing perfusion and drug delivery in pancreatic tumors appear to be TGFβ-regulated but are capable of rapid adaptation to prolonged TβR-I blockade.

TβR-I antagonist induces shift of myCAF:ICAF ratios, which govern tumoral perfusion

To identify cellular elements governing tumoral perfusion we examined the phenotypical features of cancer-associated fibroblast (CAF) cell populations next (25, 29). Recent studies examining the heterogeneity of different CAF subtypes in pancreatic tumors suggested that the SMA-positive myelofibroblastic phenotype of CAFs (myCAF) is associated with restricted intratumoral perfusion (29). Whereas TGFβ-dependent myCAFs have been found densely surrounding glandular cancer elements, the JAK/IL1-driven inflammatory phenotype fibroblasts (iCAF) are more loosely distributed among cancer glands and tumor cells (29, 30). To connect CAF phenotype with tumoral perfusion, we first treated KP16 animals with LY364947. TGFβ signaling has been shown to antagonize IL1-induced JAK/

Figure 1.

Pancreatic tumors develop resistance to TβR-I inhibition-mediated increased perfusion and drug penetration. **A**, Representative IHC stains of KP16 tumors with anti-collagen I (top), smooth muscle actin (SMA; middle), and Ki67 (bottom) treated for 2 days (left) and 14 days (right). Quantifications of $n \geq 4$ tumors per group derived from computer-based scanning on right. Scale bars, 300 μm . **B**, Tumoral dextran perfusion after 2 days of vehicle and TβR-I inhibitor treatment. Left, tiled confocal microscopy images showing intratumoral FITC-dextran distribution of vehicle and LY364947-treated animals. Tumor edges outlined in red, images of spleens used for normalization shown in insets. Quantifications of indicated treatment groups shown on right. **C**, Perfusion-weight abdominal MRI imaging of KP16 tumors. Panel shows MRI images of a single KP16 animal (1) pretreatment, prior to contrast, (2) pretreatment, at 5 minutes after contrast injection, (3) after treatment with TβR-I inhibitor, prior to contrast, and (4) after treatment, at 5 minutes after contrast injection, measured tumors outlined in red. Quantification of mean contrast intensity within tumors after treatment with vehicle and LY364947 ($n = 4$ per group) on right, all tumor intensity measurements normalized to paraspinous skeletal muscle. Error bars, SEM. **D**, Lectin perfusion of KPC tumors. Left, coimmunofluorescent confocal microscopy images of KPC tumors stained with anti-CD31 (green) and fluorolabeled lectin (orange) labeling intratumoral erythrocytes. Quantification of ratios of CD31⁺ vessels containing lectin signal versus nonperfused CD31⁺ vessels on right. Scale bars, 200 μm . **E**, Intratumoral gemcitabine levels after 2 days (left) and 14 days of TβR-I inhibition, $n = 8$ KP16 animals per group. dFdC, 2',2'-difluorocytidine; dFdU, 2',2'-difluorodeoxyuridine; dFdCTP, 2',2'-difluorodeoxycytidine-5'-triphosphate.

STAT3 signaling required for the inflammatory phenotype of CAFs (29). In agreement with these data, T β R-I inhibition decreased tumoral myCAF and increased iCAF fractions in treated animals compared with vehicle control (Fig. 2A). This shift was transient, as myCAF and iCAF fractions reverted to the baseline levels after 7 days of T β R-I inhibition in KP16 mice and 2 weeks in KPC mice (Fig. 2A; Supplementary Fig. S1D and S1E). The return to baseline myCAF and iCAF fractions upon prolonged treatment was associated with the reemerge of SMAD2 phosphorylation levels in the SMA⁺ myCAF population detected by immunocytochemical p-SMAD2 SMA costaining (Supplementary Fig. S1F). Next, we examined whether T β R-I inhibition-induced changes in myCAF-to-iCAF fractions are associated with changes in spatial distribution of CAFs. IHC staining of vehicle-treated tumors revealed tumor glandular structures more frequently tightly surrounded by SMA⁺ cells (Fig. 2B). This periglandular location of SMA⁺ cells was less common in tumors treated with the T β R inhibitor for 2 days but increased upon prolonged treatment (Fig. 2B and C). To examine whether the effect of the low-dose T β R-I inhibitor on CAF distribution involves changes in the EMT differentiation state of the tumor cells, we evaluated expression of the epithelial markers Ecadherin, cytokeratin 19, and vimentin next. There was no change in the EMT phenotype between vehicle- and T β R-I inhibitor-treated KP16 tumors (Supplementary Fig. S2A). In line with the original report of Kano and colleagues on low-dose T β R-I inhibition, flow cytometry studies conducted on pancreatic tumor digests showed that low-dose T β R-I blockade minimally suppressed SMAD3 phosphorylation in the tumoral cell compartment compared with cells in the tumor microenvironment including CD45⁺ immune cells and CAFs where low-dose TGF β inhibition effectively reduced phospho-SMAD3 levels (Supplementary Fig. S2B). To show that distinct CAF phenotypes are involved in governing tumoral perfusion, we blocked the T β R-I inhibitor-mediated induction of iCAF formation with the addition of the JAK1 inhibitor AZD4088 next. TGF β signaling suppresses JAK1/STAT3 activation essential for the iCAF phenotype (29). Flow cytometry analysis of tumors pretreated with AZD4088 for 3 days, which did not impact myCAF fractions or the total pool of intratumoral CAFs, did not show the T β R-I inhibitor-induced reduction of the myCAF fraction preventing the switch toward the iCAF phenotype observed with LY364947 treatment alone (Fig. 2D). Impeding the myCAF-to-iCAF switch was associated with lack of increased perfusion in KP16 tumors treated with the T β R-I inhibitor (Fig. 2E and F). Overall, these findings suggest that distinct CAF cell populations govern tumoral perfusion and that intratumoral perfusion levels can be influenced via manipulation of CAF phenotypes.

myCAFs harbor copy-number variations affecting genes associated with CAF activation

To provide a possible rationale and propose a mechanism which explains the temporal differences in the response to TGF β blockade in the mCAFs, we hypothesized that genes possibly affected by copy-number variations (CNV) within the myCAF population may provide resistance cues to TGF β inhibition. While considered a hallmark of the cancer compartment and not confirmed in all studies, several reports have suggested CAFs to harbor chromosomal aberrations and structural genomic variants in the form of chromosomal amplifications and losses promoting cancer cell invasion and tumor growth (31–33). Genomic instability including amplification of gene loci is a well-known mechanism of resistance to targeted therapy in cancer cells (34). To study possible CNVs and genes affected by CNVs in the CAF population of pancreatic cancers in an unbiased fashion, we first

identified CAF subpopulations via scRNA-seq (Fig. 3A and B; Supplementary Fig. S3). Next, we interrogated both CAF and ductal carcinoma populations for CNVs using the previously reported inferCNV algorithm (<https://github.com/broadinstitute/inferCNV>). Fibroblasts and ductal cells identified by scRNA-seq in normal, uninvolved pancreas were used as reference (Supplementary Table S2). Next, inferCNVs in both CAF and ductal carcinoma cells were validated with CNVs previously reported within a large high-resolution array comparative genomic hybridization (aCGH) of KPC tumors (Supplementary Table S2; 26). Surprisingly, CNVs were exclusively detected in the myCAF subpopulation and not in the other two CAF phenotypes (Fig. 3C). In comparison to ductal carcinoma cells, myCAFs were significantly less frequently affected by CNVs (Fig. 3D). There were 174 genes affected by CNVs in the myCAFs that were unique for the myCAFs and not shared with genes affected by CNVs in ductal carcinoma cells (Fig. 3E). Four out of the top seven enriched pathway in this gene set unique for myCAFs included pathways previously linked with CAF activation and CAF-induced cancer cell invasion such as focal adhesion, endocytosis, extracellular matrix receptor interaction, and MAPK signaling pathway (Supplementary Table S2; 35). Among the identified genes found to be present in at least two of the top pathways, fibroblast growth factor receptor 1 (Fgfr1), platelet-derived growth factor receptor B (Pdgfrb), and bridging integrator 1 (Bin1), were previously shown to have a pro-invasive role in CAFs or promoting cancer cell invasion and tumor growth (Supplementary Table S2; refs. 36–38).

Next, we isolated myCAFs and iCAFs from KPC tumors treated with vehicle and the T β R-I inhibitor via FACS (Supplementary Fig. S3B). Quantitative RT-PCR analysis of iCAF and myCAF markers of the two phenotypes confirmed the identity of isolated iCAF and myCAF cells (Fig. 3F). After normalization to housekeeping genes, qRT-PCR in the isolated CAF populations confirmed gene expression levels of Fgfr1, Pdgfrb, and Bin1 to be higher in isolated myCAF compared with the iCAF population affirming findings from the scRNA-seq and inferCNV analyses (Supplementary Fig. S3C). Using additional, previously described markers of CAF reprogramming, there was increased expression of the iCAF markers Il1r1, Il6, and Cxcl1 in the iCAF population isolated from KPC tumors treated with the T β R-I inhibitor for 2 days (Fig. 3G; 29). However, and in line with a rapid reversal of the induced myCAF-to-iCAF reprogramming, gene expression levels of induced iCAF markers returned to baseline upon prolonged T β R-I. On the other hand, genes affected by CNVs increased upon extended treatment with the T β R-I inhibitor in the myCAF population, an effect not observed in the iCAFs (Fig. 3H). These changes in the myCAFs were accompanied by unaltered intracellular phospho-ERK signal transduction levels with the majority of the CAF cell fraction staining positive for p-ERK on flow cytometry analysis (Supplementary Fig. S3D). Overall, these findings suggest that myCAFs in pancreatic tumors harbor unique CNVs affecting genes that have been shown to regulate CAF-mediated cancer cell progression or TGF β antagonism. Genes affected by CNVs increase in myCAFs of KPC tumors upon extended TGF β inhibition, and may lock this CAF population into a myCAF phenotype that is resistant to T β R-I blockade.

T β R inhibition in combination with gemcitabine reprograms the intratumoral immune landscape and suppresses tumor growth

To determine whether TFG β inhibition can potentiate despite the rapid adaptation of tumoral stroma the antitumoral effect of common chemotherapeutic agents, we examined the impact of T β R-I blockade in combination with gemcitabine on tumor growth and survival in

Figure 2.

CAF populations govern intratumoral perfusion. **A**, Quantification of flow cytometry analysis of KP16 tumor digests treated with vehicle or TβR-I inhibitor for 2 or 7 days determining PDPN⁺ (total CAFs; left) and SMA⁺ (myCAFs) and Ly6C⁺ (iCAFs) fractions. Representative flow cytometry plots showing PDPN⁺ cell fractions and SMA⁺ and Ly6C⁺ cells gated out of PDPN⁺ population shown on bottom. **B**, Spatial distribution of SMA⁺ cells in KP16 tumors. Left, representative IHC images after α-SMA staining. Right, quantification of ratio of tumor glands surrounded by SMA⁺ cells versus tumor glands without adjacent SMA⁺ cells. Scale bars, 200 μm. **C**, Quantification of tumor glands surrounded by SMA⁺ cells after 14 days of treatment. **D**, Quantification of flow cytometry analysis of total PDPN⁺ CAFs, SMA⁺ (myCAFs), and Ly6C⁺ (iCAFs) fractions after 2 days of treatment with LY364947, administration of JAK inhibitor AZD4088 started 3 days prior to LY364947. Ratios of Ly6C⁺-to-SMA⁺ cell fractions on right. **E**, Quantification of intratumoral localization of SMA⁺ cells after 14 days treatment. **F**, Quantification of tumor perfusion via tumor fluoro-labeled dextran intensities (normalized to spleen).

KP16 mice next. While treatment of established tumors with gemcitabine alone, or LY364947 alone, only marginally impacted tumor growth and survival, somehow surprisingly, treatment with the combination of the TβR inhibitor and gemcitabine resulted in significantly delayed tumor growth and a near doubling of median overall survival (Fig. 4A and B). In line with the suppression of tumor growth in the TβR-I inhibitor gemcitabine combination group, apoptosis rates measured as cleaved caspase 3–positive CD45⁺ cancer cell fractions

were increased and highest in the combination group. (Fig. 4C). TGFβ inhibition alone, or TβR blockade in combination with gemcitabine, did not affect vascularization of KP16 tumors after 14 days of treatment (Supplementary Fig. S4A). We then hypothesized that immunologic effects cooperatively induced by the two agents are responsible for the observed antitumor effect. TβR-I blockade in combination with gemcitabine cooperatively reduced CD4⁺FoxP3⁺ T regulatory cells (Fig. 4D), increased intratumoral CD8a⁺ T cells

Figure 3.

Gene signatures encoded by genomic variants in myCAFs are associated with CAF activation. **A**, t-distributed stochastic neighbor embedding (t-SNE) plot of all 25,760 cells of 16 KPC tumor digest, cell type annotations shown on the right. **B**, t-SNE plots of digitalSorter analysis of CAF marker genes in fibroblast, perivascular, and EMT-like cells (left) and in all cell populations (right). **C**, CNVs in CAF populations of KPC tumors. Hierarchical heatmap of inferCNVs in single cells (rows) of CAF populations (indicated on bottom). Fibroblasts identified by scRNA-seq in normal pancreas of 12 animals, 24,214 cells, were used as control. Affected chromosomes indicated on bottom, inferred copy number (log ratio) scale shown on left, losses in blue, gains in red. **D**, Large-scale CNVs of single cells (rows) of ductal carcinoma cells and CAFs of 16 KPC tumors. Ductal cells of normal pancreas were used for calculation of shown log-ratios of ductal carcinoma cells. **E**, Venn diagram indicating genes afflicted by CNVs, pathways enriched in gene expression set unique for myCAFs identified after gene set enrichment analysis (GSEA) shown on bottom (count; number of genes represented in indicated pathway). **F**, Identity of isolated mCAF and iCAF populations after FACS. qRT-PCR analysis showing gene expression levels normalized to 18S housekeeping gene of iCAF marker (*Cxcl12*, *Pil6*) and myCAF marker (*Tgfb1*, *Acta2*) genes in indicated CAF fractions. **G**, Gene expression levels of genes associated with CAF reprogramming determined by qRT-PCR in isolated CAF populations. **H**, qRT-PCR analysis of isolated CAF populations of genes afflicted by CNVs. Normalized gene expression levels of indicated genes in vehicle-treated tumors were set at 1, bars indicate relative change of mean of $n \geq 4$ tumors of shown group. Error bars indicate SEM.

(Fig. 4E), and reduced MDSCs (Fig. 4F), but had no impact on total tumor associated macrophages, dendritic cell, or intratumoral B cell infiltration (Fig. 4G). TβR-I blockade promoted M1 macrophage differentiation and countered the pro-M2 impact of gemcitabine (Fig. 4H). Examples of the employed gating strategies for the identification of individual cell populations are shown in Supplementary Fig. S4B. Similar changes were seen in KPC tumors (Supplementary Fig. S5A). To provide clinical correlates for the observed reduction of T regulatory cell and MDSC fractions in response to suppression of TGFβ signaling, we correlated TGFβ1 transcript and mRNA levels of a TGFβ response signature (TBRS)

measuring TGFβ-regulated genes with expression levels of T regulatory cell and MDSC markers in clinical specimens of pan-cancer and pancreatic cancer patients from the TCGA initiative. Defining Treg^{high} tumors by gene expression levels of the Treg markers CD25 and FoxP3 greater than the mean of all tumors of the data set ($N = 94,520$), TGFβ1 and TBRS expression levels strongly correlated with the expression of T regulatory cell (CD25, FoxP3) and human MDSC (CD11b, CD33, CD14, CD15) marker genes (Fig. 4I; Supplementary Fig. S5B) supporting the finding of reduced T regulatory and MDSC cell fractions in TβR-I inhibitor and gemcitabine-treated tumors.

Figure 4.

TβR-I inhibition and gemcitabine cooperate to suppress tumor growth and improve immune surveillance. **A**, Relative tumor growth in KP16 animals randomized to indicated treatment groups. **B**, Kaplan-Meier analysis of overall survival. **C**, Quantification of flow cytometry analysis of cleaved caspase 3-positive CD45-CK19+ cell fractions of KP16 tumor digests, representative histograms of individual treatment groups shown on right. **D**, Quantification of flow cytometry analysis of FoxP3+CD4+ T regulatory cell populations, representative histograms shown on right. **E-G**, Quantifications of flow cytometry analysis of indicated immune cell populations in KP16 tumor digests, IHC images show vehicle and TβR-I inhibitor in combination with gemcitabine-treated tumors stained with CD8α. Scale bars, 100 μm. **H**, M1 and M2 tumor associated phenotypes by flow cytometry analysis of CD86+ (M1) and CD206+ (M2) F4/80+ cell fractions of KP16 tumors. **I**, Correlation of TGFβ response signature gene set (TBRs) and TGFβ1 ligand transcripts and T regulatory cell marker expression FoxP3 and CD25 (defined as high by ≥ mean of all specimens, shown in red) in all cancer (top) and pancreas cancer (bottom) clinical specimens from TCGA cancer dataset. Correlation of high MDSC markers CD33 and CD11b and high TGFβ signaling on right.

TβR blockade in combination with gemcitabine reprograms intratumoral T regulatory and CD8 T cells

Next, we aimed to further characterize phenotypic and functional changes in immune cell populations affected by the TβR-I inhibitor gemcitabine combination. First, we isolated regulatory T cells from pancreatic tumors of animals treated with the TβR-I inhibitor, gemcitabine, or both agents, and characterized known markers of T regulatory cell phenotype on these cells (39). qRT-PCR analysis of isolated intratumoral T regulatory cells and flow cytometry analysis of tumor digests showed that TGFβ antagonism in combination with gemcitabine cooperatively reduced expression of CCR3 and CCR5 associated with the effector/memory phenotype of T regulatory cells, and upregulated CD62 L and CCR7 known to be associated with naïve T regulatory cells (Fig. 5A and B; Supplementary Fig. S6A). To examine whether the change in T regulatory cell marker profile is associated with different function, we studied the impact of T regulatory cells isolated from tumors of treated animals on tumor antigen recognition by CD8⁺ T cells. Commensurate with the immune suppressive function of T regulatory cells, the addition of T regulatory cells isolated from vehicle-treated animals impaired antigen recognition of CD8⁺ T cells cocultured with KP16 cancer cells compared with CD8⁺ T cells cocultured with cancer cells alone (Fig. 5C). T regulatory cells isolated from tumors treated with the TβR-I inhibitor gemcitabine combination showed reduced suppression of INFγ release by CD8⁺ T cells suggesting that the observed switch of T regulatory cell markers is associated with altered function. Next, we isolated CD8⁺ T cells from tumor digests and spleens and examined antigen recognition and T-cell activity. Upon coculture with KP16 pancreatic cancer cells CD8⁺ T cells isolated from tumors treated with TβR-I inhibition in combination with gemcitabine showed an approximately 5-fold increased INFγ release than CD8⁺ T cells isolated from vehicle- or either single agent-treated tumors tumor cells (Fig. 5D). Induced increased T-cell recognition was specific for intratumoral T cells and not seen on T cells isolated from spleens of same animals. CD8⁺ T cells from the combination group had elevated T-cell activation markers 4-1BB (CD137) and OX40 (CD134; Fig. 5E; Supplementary Fig. S6B), showed reduced PD-1 expression (Fig. 5F; Supplementary Fig. S6C), and elevated transcript levels of granzyme and perforin (Fig. 5G and H). To show that the survival gain was due to the observed activation of antitumor T-cell activity, we randomized tumor-bearing animals to IgG₁ isotype control, anti-CD8⁺, and anti-CD8⁺ in combination with TβR-I blockade and gemcitabine treatment next. Depletion of CD8⁺ T cells significantly reduced the survival gain provided by TβR-I blockade and gemcitabine (Fig. 5I; Supplementary Fig. S7A) and reduced the suppressive effect of the combination treatment onto tumor growth (Fig. 5J). These findings support the induction of immunogenic cooperativity of low-dose TβR-I blockade and gemcitabine in pancreatic cancer.

TβR-I blockade in cooperation with gemcitabine increases PD-L1 expression and sensitizes pancreatic tumors to immune checkpoint blockade therapy

T-cell-mediated immunotherapies, treatment with TGFβ inhibitors, or treatment with cytotoxic chemotherapy have previously been shown to elevate death cell receptor ligand 1 (PD-L1) expression on cancer cells (40, 41). TβR inhibition and gemcitabine, and to a lesser degree TβR-I blockade alone, increased PD-L1 expression on cancer cells, a finding confirmed via costaining of respective tissue sections (Fig. 6A and B). To better understand the molecular mechanisms of PD-L1 upregulation as a possible mechanism of resistance in pancreatic cancers, we examined PD-L1 expression levels in murine and

human pancreas cancer cells upon treatment next. Treatment concentrations were selected from full ten concentration dose-response curves ($\geq$ IC₉₅ values; Supplementary Fig. S7B). Treatment with TβR inhibition in combination with gemcitabine showed the largest induction of PD-L1 expression (Fig. 6C and D). To show that the observed increase in PD-L1 expression is indeed dependent on TGFβ downstream signaling, we repeated above treatment studies in a pancreas cancer reporter cell line measuring PD-L1 reporter activity. TβR-I inhibition and gemcitabine increased activity of the wild-type human PD-L1 promoter that contained 2,039 bp upstream of the PD-L1 gene transcriptional start site (TSS) several SMAD binding elements (SBE). On the other hand, no increase in PD-L1 promoter activity was observed in a mutant construct that lacked SBE elements but contained the more proximal IFNγ binding site (778 bp upstream of the TSS of PD-L1 gene). In line with the observed dependency on intact TGFβ signaling elements, TβR-I antagonism in combination with gemcitabine did not increase PD-L1 expression in SMAD4-deficient L3.6pl or TβR-II-deficient BxPC3 pancreatic cancer cells (Fig. 6E). To support that TGFβ signaling exerts inhibitory effects on PD-L1 expression in pancreas cancer cells, we knocked down TGFβ signaling effectors pSMAD2 and pSMAD3 during treatment. Silencing of the TβR downstream mediators SMAD3, but not SMAD2, cooperated with TβR inhibition and further enhanced induction of PD-L1 expression (Fig. 6F). While single-agent PD-L1 inhibition, in line with clinical experience in pancreas cancer, did not show any benefit in comparison with vehicle-treated control, the addition of PD-L1 checkpoint blockade to TβR-I in combination with gemcitabine further extended survival (Fig. 6G). Thus, tumoral PD-L1 expression governed by TGFβ signaling appears to be a mechanism of resistance to TβR-I inhibition in combination with gemcitabine in tumors with intact TGFβ signaling and the addition of PD-L1 immune checkpoint blockade appears to further improve outcome of the combination approach.

Discussion

Successful systemic cancer therapy requires effective drug transport to cancer cells in all regions of the tumor (16, 42). Intratumoral biodistribution is hereby determined by convective drug flux and drug transport of diffusion which are a function of (i) the biophysical properties of the administered agent, (ii) the intratumoral vasculature, and (iii) the extravascular space (42). Cellular partitions might require drug solutes to cross lipid bilayers which can significantly reduce intratumoral biodistribution (9). To circumvent cellular partitions drug solutes might need to follow tortuous transport pathways surrounding these cells, which might be opposite to diffusion gradients or convective flux, which reduces, or abolishes, effective drug penetration (42). In our study, we focused on CAFs as a major cellular element of such physical barriers in the pancreas cancer stroma. Two independent studies recently identified two, possibly interconvertible, main phenotypes of CAFs (29, 30). Our findings that TGFβ inhibition shifts the myCAF-iCAF ratio is in line with the original description and studies in these CAF populations which showed that myCAFs are dependent on TGFβ signaling and that the release of TGFβ-mediated repression of interleukin 1 receptor (IL1R) expression levels increases expression of iCAF markers like Ly6C and activates JAK1/STAT3 signaling in these cells (29, 43). Importantly, the myCAF-to-iCAF shift was associated with a spatial redistribution of CAFs in the TME releasing the tight surrounding of cancer glands and islets by SMA⁺ myCAFs, which is likely induced by tumoral cell-derived paracrine TGFβ production, towards the more interspersed, more loosely

Figure 5.

TβR-I antagonism and gemcitabine cooperate to improve antitumor T-cell function. **A**, qRT-PCR analysis of indicated genes in T regulatory cells isolated from KP16 tumors of indicated treatment groups. Gene expression levels in cells isolated from vehicle-treated tumors was set 1; graph indicates mean of $N = 4$ animals per group, measured in triplicates. Error bars, SEM. **B**, Quantification of flow cytometry analysis of KP16 tumors treated with vehicle and TβR inhibitor in combination with gemcitabine. Fraction of % positive cells FoxP3⁺CD4⁺ cells in vehicle-treated group was set at 1, $N = 4$ per group. **C**, Coculture assay examining T regulatory cell function on CD8⁺ T-cell function. T regulatory cells isolated from KP16 tumors were added to CD8⁺ T cells isolated from spleen of tumor-bearing animals and KP16 cancer cells, IFN γ release was measured by ELISpot assays. Quantification of T regulatory cell impact on IFN γ release on right, number of ELISpots in control group of CD8 T cells and KP16 cancer cells was set at 1, $n = 3$ independent experiments, in triplicates. **D**, Induction of IFN γ secretion of CD8 T cells isolated from KP16 tumors and spleens after coculture with KP16 cells, quantification on right. **E** and **F**, Quantification of flow cytometry analysis of intratumoral CD8 T cells. **G**, qRT-PCR analysis of indicated genes in total tumor RNA. $n = 4$ per group, in triplicates. **H**, qRT-PCR analysis of indicated genes in CD8 T cells isolated from KP16 tumors. **I**, Kaplan-Meier analysis of KP16 animals treated with TβR inhibitor and gemcitabine after CD8⁺ T-cell depletion (red curve) or isotype control (purple curve). **J**, Relative tumor growth in T-cell-depleted versus isotype IgG control-treated KP16 mice.

Figure 6.

PD-L1 expression is a mechanism of resistance to T β R-I inhibition and gemcitabine. **A**, Quantification of flow analysis of CK19⁺ PD-L1⁺ cell fractions of KP16 tumor digests, representative histograms of individual treatment groups shown on right. **B**, Confocal microscopy images of KP16 tumors treated with vehicle and T β R-I inhibition in combination with gemcitabine costained with α -cytokeratin 19 (CK19; red) and PD-L1 (green). Colocalization of CK19 (red) and PD-L1 (green) generating yellow emission. Laser intensity profiles shown on right after linear scanning of random tissue section measuring intensity (fluorescence intensity, y-axis) vs. distance (in μ m; x-axis) shows cells labeled both red (CK19) and green (PD-L1; blue; DAPI) indicating colocalization (white arrows). Quantification of CK19⁺ PD-L1⁺ cell fractions of $n = 4$ tumors on right. **C**, qRT-PCR analysis of PD-L1 expression in KP16, PANC1, L3.6pl, and BxPC3 pancreatic cancer cell lines. Expression levels in vehicle-treated cells was set at 1, $n = 3$ independent experiments, in triplicates. **D**, Quantification of flow cytometry analysis of PD-L1⁺ cell fractions. $n = 3$ independent experiments. **E**, Reporter assay of PD-L1 expression. Reporter activity in PANC1 cells transfected with SMAD binding element (SBE)-containing wild type PD-L1 reporter (left) and mutant PD-L1 reporter lacking SBE elements after normalization to cotransfected control, response to IFN γ shown as control. **F**, Impact of loss of TGF β effectors SMAD2 and SMAD3 on PD-L1 expression. qRT-PCR products in PANC1 cells visualized by gel electrophoresis and ethidium bromide staining after knockdown with scramble, SMAD2, and SMAD3 siRNA. Quantification of qRT-PCR analysis (middle) and flow cytometry analysis (bottom) of PANC1 cells subject to indicated treatments and silenced by scramble, SMAD2, and SMAD3 siRNA. $n = 3$ independent experiments in triplicates. **G**, Kaplan-Meier analysis of survival of KP16 animals treated with T β R inhibitor and gemcitabine (red curve) and in combination with PD-L1 antibody (purple curve).

connected iCAF phenotype (43). The myCAF-to-iCAF switch was accompanied by an increase in perfusion and intratumoral drug delivery and it is likely that the lack of cellular partitions in the form of tight, interconnected myCAFs surrounding tumor glands opens access areas of drug transport to additional tumor areas. Blockade of the TGF β inhibitor-induced myCAF-to-iCAF switch via antagonism of T β R-I inhibitor-mediated JAK1 signaling activation in the iCAF population prevented the shift from the TGF β antagonist-induced increase in perfusion (29). Surprisingly, suppression of myCAFs and the increase in tumoral perfusion was short-lived. Induced changes

towards a greater iCAF phenotype upon T β R-I were short-lived, and the loss of reprogramming function of TGF β inhibition was triggered by endogenous resistance mechanisms in the myCAF population. Single-cell CNV analysis identified unique CNVs in the myCAF population and genes affected by CNVs included known regulators of CAF activation, CAF-mediated cancer cell invasion, or antagonists of TGF β signaling (36–38). Similar to mediators of resistance to targeted therapy in cancer cells afflicted by chromosomal amplifications and CNVs, like amplification of the c-MET oncogene in EGFR-mutant non-small cell lung cancer, the elevated gene transcript levels

affected by CNVs in myCAFs escaped reprogramming function of TGF β inhibition and downstream effectors like p-ERK of these genes reported to mediate a CAFs a procancer phenotype in other malignancies remained high and possibly prevented a sustained switch of the myCAF phenotype (35). While we have not targeted these putative mediators of resistance to TGF β inhibition in the myCAF subpopulation, the elevated expression levels upon extended T β R-I inhibition combined with a lack of suppression of ERK activation make us believe that genomic causes should be considered as possible mediators of resistance to pharmacologic manipulation of CAF populations in pancreas cancer. In this regard, it is tempting to speculate whether myCAF-to-tumor gland, or myCAF-to-ICAF ratios can select tumors in the future with poor perfusion possibly indicating increased risk of therapy failure.

We were initially surprised by the cooperative effect of TGF β inhibition and gemcitabine, when the effects of TGF β i on tumor stroma were transient. Pleiotropic TGF β signaling in PDAC has stage- and cell-specific roles which are, in part, dichotomously opposing (44). In tumoral cells of pancreas cancer TGF β signaling has a well-investigated tumor-suppressive function; loss of T β R-II or SMAD4 has been shown in elegant genetically engineered murine models of pancreas (GEMM) to cooperate with KRAS activation to generate PDAC, and in the homozygous configuration to accelerate PDAC progression compared to heterozygous loss (45, 46). However, at late, more evolved stages, TGF β signaling in the stroma exerts a tumor-promoting, pro-EMT, metastasis-facilitating, and overall immune suppressive role and in line with levels of TGF β ligand isoforms, which are highly expressed in human PDAC, being associated with adverse clinical outcome in PDAC patients (44). To exploit these tumor stage- and cell type-specific effects has been a major challenge and is likely one of the main reasons for the modest activity of anti-TGF β therapy observed in clinical trials so far. In preclinical studies, Hezel and colleagues observed in both early and late Kras-p53lox/+ mice increased Ki67 expression and worse clinical outcome in animals treated with a pan-TGF β blocking mAb compared with isotype control (47). Huang and colleagues observed similar effects in KIC and KPC mice treated with a T β R-II blocking antibody, but not in a mouse model with disrupted TGF β signaling in the epithelial cancer cells (48). The lack of increased Ki67 expression and accelerated tumor growth in our study thus seems to contradict these previous findings. However, tumors in our study were more evolved where the tumor-restricting effect of TGF β signaling inhibition of stromal cells in the evolved tumor microenvironment (TME) assumes a greater role compared to early, and in particular pre-invasive lesions where in the absence of an evolved TME TGF β signaling exerts a greater tumor-suppressive function. For example, while in our study enrolled mice had ultrasound-confirmed evolved pancreatic tumors of 4–6 mm for KP16 (= KIC) and 5–10 mm for KPC mice, KPC mice enrolled in the study of Hezel and colleagues with late tumors at 9 weeks of age are likely to not infrequently have pancreatic intraepithelial neoplasia (PanIN) and preinvasive disease and no advanced tumors (47). Huang and colleagues started to treat KIC (= KP16) mice at 6 weeks of age where possibly the majority of animals has no detectable tumors or very small tumors, and KPC mice at 90 days where a proportion of mice will not have developed advanced tumors (48). Another important difference to above studies, is the use of a low-dose TGF β inhibitor regimen of 1 mg/kg every other day compared with the administration of 20 mg/kg regimen commonly used in studies with LY364947 or galunisertib (LY2157299) a similar small molecule. Kano and colleagues elegantly showed in xenotransplantation models of pancreas and gastric cancer low-dose TGF β inhibition predominantly suppresses

TGF β signaling in stromal cells but not in tumoral cells or vasculature in normal, uninvolved organs (22). Indeed, p-SMAD3 levels were only marginally changed in the tumoral cell population compared with a significantly greater reduction of SMAD3 phosphorylation in immune cells and interstitial CAF cells, a finding we were able to reproduce in the employed GEMMs. While we do not know the reason for the different sensitivity to TGF β inhibition and cannot rule out that noncanonical TGF β signaling might have been affected in the cancer cells, the lack of p-SMAD3 suppression is in line with the absence of increased Ki67 expression or a change in the EMT phenotype of the epithelial cancer cells.

TGF β antagonism and gemcitabine therapy are both known to impact the immune landscape of pancreas cancer. Despite the transient impact of TGF β inhibition on CAF phenotype, tumoral perfusion, and rapid reformation of the stromal barrier, low-dose TGF β blockade coadministered with gemcitabine was able to reprogram immune cell populations in the TME, which were able to mount T cell-mediated antitumor responses. While T β R-I inhibition in combination with gemcitabine also reprogrammed TAMs toward an inflammatory, M1-like phenotype, we selected T regulatory cells for further investigation to explain the cooperative action of the two agents. Tumors educate T regulatory cells from a naïve toward an effector memory phenotype (49), and T β R-I blockade and gemcitabine suppressed several of the T regulatory cell markers associated with the immune suppressive, effector memory phenotype. The tumor-restricting effect induced by the reduction and reprogramming of CD4⁺ T regulatory cell function seems to be in contrast with a recent report where genetic depletion of CD4⁺CD25⁺FoxP3⁺ regulatory T cells showed an acceleration of pancreatic cancer formation (50). The authors show that the lack of TGF β derived from the depleted T regulatory cells induced a number of protumor changes (50). These differences to our findings might be explained by the heterogeneity of the intratumoral T regulatory cell population (51); for example, not all CD4⁺ T regulatory cell populations suppress T effector cells and hinder host immune responses against cancer. CCR5⁺ or LAG3⁺ T regulatory cell subpopulations, which were observed to show a disproportionately large reduction compared with other effector/memory subsets in the T β R-I inhibitor gemcitabine-treated tumors, are enriched in the TME of cancers, and T regulatory cells with high CCR5 or LAG3 expression have high immune-suppressive function, whereas CCR5⁺ and LAG3⁺ T regulatory cell-deficient models showed slowed disease progression (52, 53). Thus, the checkpoint profile on T regulatory cells might provide future leads for immunoncology combination studies. The identified mechanism of resistance of PD-L1 upregulation is on the other hand known from other cancer immunotherapies which elicit anti-tumor T cell responses via INF γ release (40). Of note, the upregulation of PD-L1 expression on cancer cells upon TGF β inhibition by pancreatic cancer cells is hereby different to other solid organ cancers, like for example non-small cell lung cancer where TGF β agonism has been shown to upregulate PD-L1 (54).

In summary, this study gives credence to a growing chorus of reports which ask for a re-thinking of a unidirectional concept of stromal therapy focused in overcoming the physical barrier of the stroma to correct hypoperfusion and improve drug delivery to enhance tumoral cell death and tumor control. Here we show that pharmacologic manipulation via TGF β inhibition of CAF phenotypes can increase tumoral perfusion. While rapid escape from TGF β antagonism in the myCAF subpopulation returns perfusion to baseline levels, immunologic cooperativity independent of genotoxic action of gemcitabine between the two agents suppresses tumor growth. Hence,

stromal-targeting strategies need to take the evolving understanding of interdependencies of stromal cell populations and subpopulations into account to not miss evolving therapeutic opportunities for future improved therapy.

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