Clinically relevant orthotopic pancreatic cancer models for adoptive T cell transfer therapy

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ABSTRACT
Background Pancreatic ductal adenocarcinoma (PDAC) is an aggressive tumor. Prognosis is poor and survival is low in patients diagnosed with this disease, with a survival rate of ~12% at 5 years. Immunotherapy, including adoptive T cell transfer therapy, has not impacted the outcomes in patients with PDAC, due in part to the hostile tumor microenvironment (TME) which limits T cell trafficking and persistence. We posit that murine models serve as useful tools to study the fate of T cell therapy. Currently, genetically engineered mouse models (GEMMs) for PDAC are considered a "gold-standard" as they recapitulate many aspects of human disease. However, these models have limitations, including marked tumor variability across individual mice and the cost of colony maintenance.
Methods Using flow cytometry and immunohistochemistry, we characterized the immunological features and trafficking patterns of adoptively transferred T cells in orthotopic PDAC (C57BL/6) models using two mouse cell lines, KPC-Luc and MT-5, isolated from C57BL/6 KPC-GEMM (KrasLSL-G12D/+p53−/−) and KrasLSL-G12D/+p53−/−, respectively.
Results The MT-5 orthotopic model best recapitulates the cellular and stromal features of the TME in the PDAC GEMM. In contrast, far more host immune cells infiltrate the KPC-Luc tumors, which have less stroma, although CD4+ and CD8+ T cells were similarly detected in the MT-5 tumors compared with KPC-GEMM in mice. Interestingly, we found that chimeric antigen receptor (CAR) T cells redirected to recognize mesothelin on these tumors that signal via CD3ζ and 41BB (Meso-41BBζ-CAR T cells) infiltrated the tumors of mice bearing stroma-devoid KPC-Luc orthotopic tumors, but not MT-5 tumors.
Conclusions Our data establish for the first time a reproducible and realistic clinical system useful for modeling stroma-rich and stroma-devoid PDAC tumors. These models shall serve an in-depth study of how to overcome barriers that limit antitumor activity of adoptively transferred T cells.

WHAT IS ALREADY KNOWN ON THIS TOPIC
⇒ Genetically engineered mouse models (ie, KPC-GEMM) used for pancreatic ductal adenocarcinoma (PDAC) research often require lengthy, variable time periods for tumor development and costly maintenance.
⇒ To combat this barrier, we present a reproducible alternative: orthotopic implantation of genetically defined syngeneic murine cell lines into the pancreas of C57BL/6 mice.

WHAT THIS STUDY ADDS
⇒ The models presented demonstrate applicability in evaluating new therapies for PDAC, such as adoptive T cell transfer therapy directed against mesothelin.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY
⇒ This work provides important insight into optimizing preclinical models for testing cell therapy approaches.

INTRODUCTION
Pancreatic ductal adenocarcinoma (PDAC) is a deadly malignancy. Only 12% of patients experience an overall 5-year survival rate. PDAC is projected to become the second leading cause of cancer-related death by 2030.1, 2 With limited therapeutic options, surgery remains the only curative approach. Unfortunately, early-stage disease is usually not detected, resulting in most individuals (80% of patients) being diagnosed with locally advanced or metastatic disease. This diagnosis renders them ineligible for surgical resection.3 Current standard of care includes cytotoxic chemotherapies, such as FOLFIRINOX (a combination therapy including FOLinic acid, Fluorouracil, IRINotecan, and OXaliplatin) or gemcitabine and nab-paclitaxel, which result in only incremental improvements to survival.4 Similarly, immunotherapy has not impacted the disease course, aside from rare subsets of patients harboring microsatellite instable tumors.5 It is dire to better illuminate the complex biology of PDAC and support efforts in developing new immune-based therapeutics for patients.

PDAC harbors a tumor microenvironment (TME) consisting of abundant non-neoplastic...
cells that crosstalk with PDAC tumors to produce a dense, desmoplastic stroma. Stroma accounts for most of the tumor (~90% by mass), and the degree of desmoplasia in PDAC tumors is linked to limited efficacy of cytotoxic chemotherapy and negative survival outcomes.\(^5\) Prior efforts to target desmoplasia using broad-acting agents such as PEG-hyaluronidase have not been met with clinical success,\(^7\)\(^8\) prompting the need to investigate the complex biology of the TME. In PDAC, the stroma is fueled by cancer-associated fibroblasts (CAFs), which produce extracellular matrix (ECM) constituents, such as collagen, that increase fibrosis.\(^9\) Adding to this complexity is the heterogeneous origin and function of CAFs, which have context-dependent roles in shaping the PDAC TME and response to therapy.\(^10\)–\(^13\) Notably, CAFs produce a multitude of soluble factors that influence immune cell composition in PDAC tumors, and compromise the infiltration, survival, and function of cytotoxic lymphocytes.\(^14\)–\(^16\) Combining these immune features with a low tumor mutation burden and elevated inhibitory immune checkpoint ligands, PDAC is considered an “immune desert.”\(^17\)\(^18\) Taken together, the dominant stromal features of PDAC limit vascularization, access of pharmacological agents, and infiltration of both endogenous and adoptively transferred immune cells into the tumors.\(^19\)

Identifying effective strategies to overcome these redundant stromal barriers could have marked impact, particularly in applying immunotherapy to PDAC. This idea is especially relevant in the development of cellular therapies in advanced disease. The efficacy of adoptive T cell transfer (ACT) therapy in PDAC is hindered by numerous factors, including poor persistence and limited access or trafficking of transferred T cells into the tumors. For instance, a phase I clinical trial treated patients with lentiviral-encoded, mesothelin-specific chimeric antigen receptor (CAR) T cells and conferred stable disease in 11 of 15 patients with PDAC, ovarian carcinoma, or malignant pleural mesothelioma. Yet the efficacy was limited, likely due to poor infiltration and persistence of immune cells in the TME.\(^20\) ACT therapy studies with CAR T cells targeting other distinct tumor antigens have produced similar results, marked by nominal access of T cells into the tumors.\(^21\) Therefore, the ability to accurately model the cellular composition of the TME and its interplay with the immune system is crucial to developing effective therapies for PDAC.

We posit that murine models are useful surrogates for accelerating preclinical efforts to improve PDAC therapy. Historically, genetically engineered mouse models (GEMMs) with oncogenic \(Kras\) have been generated to concurrently harbor alterations in \(T\text{ip}53\), including those with hot spot, loss of function (LOF) mutations, or others completely null for \(T\text{ip}53\). Collectively, these GEMMs have informed our understanding of the TME and been dependable tools for studying the complex aspects of this disease in an immune-competent setting. However, these “gold-standard” GEMMs have obvious limitations for rapid therapeutic studies, including cost, variable disease onset, and extended time to tumor development. Over time, these GEMMs have given rise to useful syngeneic cell lines that can be orthotopically implanted into the pancreas of C57BL/6 mice to generate organotropic tumors. These derivative models are not in and of themselves spontaneously arising, but do offer the advantage of rapidly generating large cohorts of tumor-bearing mice to minimize variable disease onset for preclinical studies. Although derivative cell lines are genetically very similar, they often possess variability in progression. These data imply that subtle differences in the resulting TME may occur between models, and that these differences could be exploited for approximating PDAC stromal heterogeneity. We hypothesized that syngenic PDAC tumor lines produce distinct stromal features when grown orthotopically in vivo, which in turn impact the access of immune cells to infiltrate tumors. We found that distinct stromal features of PDAC tumor models can differentially influence T cell trafficking and function in the context of ACT therapy.

**MATERIALS AND METHODS**

**Cell lines and reagents**

KPC-Luc cells (a gift from Dr Craig Logsdon, MD Anderson Cancer Center) were derived from the \(Kras\)\(^{LSL-G12D}\), \(T\text{ip}53^{−/−}\), Pdx1-cre spontaneous tumor model and express an enhanced firefly luciferase construct.\(^22\) Cells were cultured with Dulbecco’s Modified Eagle Medium DMEM (Gibco \#11965092) with 10% fetal bovine serum (FBS) (GeminiBio \#900-208), 10\(\mu\)M L-glutamine (Corning \#25-005 CI), and Antibiotic:Antimycotic Solution (GeminiBio \#50-753-3020). MT-5 cells, derived from the \(Kras\)\(^{LSL-G12D}\), \(T\text{ip}53^{−/−}\), Pdx1-cre spontaneous tumor model, were a gift from Dr David Tuveson (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) and were cultured with RPMI-1640 (Gibco \#11875093) with 10% FBS, 10\(\mu\)M L-glutamine, and Antibiotic:Antimycotic Solution. MT-5 cell lines were also transduced to stably express luciferase using Lentifact (GeneCopoeia \#LPP-HLUC-Lv105-025-C) lentiviral vectors of firefly luciferase, according to the manufacturer’s protocol. Single-cell clones of transduced cells were selected and expanded for in vivo validation. Briefly, cell populations were diluted to 0.5 cells per 200\(\mu\)L and plated in a 96-well plate. Each single-cell colony was transferred to T25 flasks and allowed to passage several times to allow for stable colony formation. The cells were then subcutaneously injected into the flank of C57BL/6 mice. Bioluminescence intensity (BLI) was measured using the IVIS system (described in the following section). Colonies with detectable luciferase expression that displayed exponential longitudinal growth subcutaneously were then implanted orthotopically in the pancreas of mice for further study.

**In vivo studies**

For orthotopic experiments, \(2×10^{3}\) murine PDAC cells were orthotopically implanted into the tail of the pancreas...
of 6-week to 8-week-old female 16 C57BL/6 mice (n=8 per cell line; Strain 000406, The Jackson Laboratory). After 10 days, tumor establishment was verified via BLI using the In Vivo Imaging System (IVIS) (PerkinElmer #124262). Briefly, the mice were injected with 180 μg/mL of 15 mg/mL D-luciferin (Biosynth #L-8220), dissolved in phosphate-buffered saline (PBS), and imaged using an open filter setting after 6 min. For model characterization and survival studies, the animals, on reaching humane endpoint criteria, were euthanized by CO2 asphyxiation, followed by cervical dislocation. For adoptive cell transfer therapy studies, the mice (n=20, 10 per cell line) were randomized such that the animals in each treatment group had comparable tumor sizes. Subsequently, total body irradiation (TBI) was performed using 5 Gy of radiation from a SmART+ X-ray Irradiator (Precision X-Ray) on day 10 postsurgery, followed by ACT therapy via tail vein injection 24 hours later (7.6×10^6 mesoCAR+ T cells per mouse).\textsuperscript{25}

**T cell transduction**

All experimental T cells were harvested from the spleens of female B6.PL-Thy1.2/CD45.2 (Strain 000046, The Jackson Laboratory) according to previously described protocols.\textsuperscript{24} Lymphocytes derived from congeneric mice were used for ACT therapy studies to distinguish donor CAR T cells (Thy1.1 (CD90.2)) from intrinsically host lymphocyte populations (Thy1.2 (CD90.2)). CD3\(^+\) T lymphocytes were negatively selected for using the EasySep Mouse T Cell Isolation Kit (Stemcell Technologies #19851) and were cultured in RPMI containing L-glutamine (Corning #21-040-CV), 10% heat-inactivated FBS (Atlas Biological #FS-0500-AD), 1 mM sodium pyruvate (Gibco #11360070), 1 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) (Gibco #15630080), 1X MEM Non-Essential Amino Acids (Gibco #111400-050), and penicillin-streptomycin (Corning #30-002-CI). αCD3/αCD28 magnetic beads (Dynabeads #114560D) were used according to the manufacturer’s protocols and administered at a 1:1 bead to T cell ratio. One hundred IU/mL rhIL-2 (National Institute of Health (NIH) repository) were added every 2 days, or as needed for 5 days, before initiating treatment. Best practices for interleukin-2 (IL-2) concentration, bead activation, and culture conditions were based on recommendations in the literature.\textsuperscript{25} MesoCAR T cells were transduced with a chimeric anti-mesothelin single-chain variable fragment (scFv) fusion protein containing the T cell receptor CD3\(^\varepsilon\) signaling domain. CAR vector generation has been described for both human and mouse-specific mesothelin-binding scFVs.\textsuperscript{26,27} The mesoCAR vector was generously provided by Dr. Carl June (University of Pennsylvania). CAR expression post-transduction was validated and quantified using a flow cytometry antibody specific to the human F(ab\(^\prime\))\(_2\) fragment (Jackson Immunolab #109-606-006).

**Western blot**

Proteins were extracted from cell lysates using mechanical dissociation via sonication, followed by suspension in Radioimmunoprecipitation assay (RIPA) lysis buffer containing 1% phenylmethylsulfonyl (Life Technologies #36978) and 1% phosphatase and protease inhibitor cocktail (Cell Signaling #5870). Protein concentration was determined using the Pierce BCA Protein Assay Kit (ThermoFisher #23227). Western blots were performed as previously described.\textsuperscript{28} The primary antibody used to detect mesothelin was anti-mouse-mesothelin (Abcam #ab213174) and the secondary antibody was anti-rabbit IgG, Horseradish peroxidase (HRP)-linked (Cell Signaling #7074). Anti-β-actin antibody was used as a loading control (Abcam #ab8226).

**Flow cytometry**

Murine tumor tissue, spleen, and blood samples were collected for immunophenotypic analysis by flow cytometry. The organs were first mechanically digested by mincing the tissue and then stained using a 70 μm filter (Fisher Scientific #08-877-1). Cell suspension was then chemically digested by incubation at 37°C for 30 min with collagenase (Roche #1108886001), dispase (Stemcell Technologies #07923), and liberase enzymes (Sigma-Aldrich #54119001). Single-cell suspensions of each organ were incubated with antibodies (table 1) in the dark at room temperature for 10 min, washed, and fixed in FACS Buffer containing 2% FBS and 4% paraformaldehyde (Sigma-Aldrich #95-30525-89-4). Zombie Aqua Fixable (BioLegend #423101) or Zombie UV viability dye (BioLegend #423107) was used to detect live cells. Samples were measured on a Cytex Aurora flow cytometer (Cytex Biosciences). Between 10×10^4 and 10×10^6 events were collected per sample and analyzed using FlowJo V9 software (Becton Dickinson). For CD45\(^+\) immune infiltration analysis, MT-3 and KPC-Luc tumor samples were harvested from the mice on days 16 and 19, respectively, postimplantation. The samples were normalized for comparison analysis by downsampling to a limit of 40,000 single cells prior to the selection of live and CD45\(^+\) cells.

**Immunohistochemistry**

Formalin-fixed paraffin-embedded mouse tumors from in vivo mouse experiments were subject to immunohistochemistry (IHC) analysis as conducted by Emory’s Cancer Tissue and Pathology Shared Resource. Sections from mouse tumors that did not render large enough tissue size were excluded from further downstream analyses. 4–5 μm slices were individually stained with hematoxylin (Sigma #517-28-2) and picrosirius red (Abcam #ab150681), and primary antibodies (table 1) were developed using DAB (3,3′-diaminobenzidine) substrate (Vector Laboratories #SK-4100). Slides were scanned using an Olympus Nanozoomer whole slide scanner. Images were analyzed using Qupath software (qupath.github.io). The total number of cells was quantified within a specified tissue area, and threshold detection
was used to count the number of cells positive for a given antibody signal. For picrosirius red, threshold detection was used to quantify the positive area (µm<sup>3</sup>) of the total tumor tissue area. Multiplex immunofluorescence staining included 4′,6-diamidino-2-phenylindole (DAPI) (PerkinElmer #CS1-0127-2ML), CD4, and CD8 primary antibodies (table 1), followed by Opal 690 and Opal 520 conjugated secondaries (PerkinElmer), respectively. Images were acquired using a Roche BenchMark ULTRA IHC/ISH System autostainer.

### Gene expression analysis
Pancreatic and lung tumor gene expression and somatic mutation data were obtained from The Cancer Genome Atlas (TCGA) ([https://www.cancer.gov/tcga: PDAC]<sup>30</sup>; lung adenocarcinoma (LUAD)<sup>31</sup>; and lung squamous cell carcinoma (LUSC)<sup>32</sup>). Pancreatic tumor cases were limited to the 150 samples previously established as ductal adenocarcinomas (PDAC). In total, 483 samples of LUSC and 530 samples of LUAD with both mutation and expression data were used. Mutation annotation format (MAF) files were used to identify samples with the TP53 R175H hotspot mutation. All other mutations were categorized as LOF, and samples with other mutation data that included no mutation in the TP53 gene were considered wild-type. Expression values were log2-transformed, then the average was computed among established normal and activated stroma gene sets for each sample ([https://github.com/rmoffitt/pdacR]<sup>33,34</sup>). Comparisons between TP53 mutation status were performed using Welch’s two sample t-test comparing the average expression of the LOF group with the hotspot group. Analysis was run in R V.4.2.1 with the stats package ([https://www.R-project.org/](https://www.R-project.org/)).

### Statistical analysis
Power analysis was used to determine the sample size of at least eight mice per group (80% power for an effect size of at least 1.5, assuming 5% significance level and a two-sided test). Statistical analyses were performed on Prism.

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IHC, immunohistochemistry; α-SMA, alpha-smooth muscle actin.
RESULTS
Syngeneic models differentially impact mouse survival and tumor composition

Orthotopic implantation of tumor cells into the pancreas of immune-competent mice represents a practical approach for testing immunotherapy in vivo. We surmised that these models would recapitulate key stromal and immune features of the standard KPC-GEMM (Kras$^{G12D}$, Trp53$^{R172H/+}$) while permitting consistency in tumor burden. Survival studies were conducted to assess progression variability between distinct, KPC-GEMM-derived PDAC tumor lines postorthotopic implantation. KPC-Luc and MT-5 tumors were used for these studies (figure 1A). These tumors were derived from two different KPC-GEMMs, each driven by oncogenic Kras and Trp53 alterations. Trp53 alteration differed slightly between the cells: the KPC-Luc cells harbor a null Trp53 ($Trp53^{-/-}$) and the MT-5 cells have a hotspot inactivating mutation ($Trp53^{R172H/+}$). Given these clinically relevant genetic profiles, we anticipated similar phenotypic properties of tumors would be evident on implantation in vivo. Orthotopic KPC-Luc was a more aggressive model, with a median survival of mice at 23 days, while MT-5-bearing mice survived a median time of 36 days (figure 1B). H&E staining revealed that MT-5 tumors closely mirrored the

Figure 1 Orthotopic PDAC tumors recapitulate the TME of GEMM tumors. (A) Experimental schematic of cell isolation and orthotopic longitudinal survival study. Immortalized cell lines were isolated from KPC-GEMMs. (B) Survival kinetics of immunocompetent mice bearing KPC-Luc or MT-5 orthotopic PDAC tumors (n=10 per group). One-way ANOVA: *$p<0.05$. ANOVA, analysis of variance; GEMM, genetically engineered mouse model; PDAC, pancreatic ductal adenocarcinoma; TME, tumor microenvironment.
Dense stroma correlates to reduced immune infiltration in orthotopic PDAC tumors

Stromal features were evaluated across the two orthotopic models and compared with established KPC-GEMM tumors from mice at 3–5 months of age via IHC. We hypothesized distinct stromal features that influence disease progression would be clearly identified and limit access of lymphocytes into the tumors. To assess this idea, the stromal composition was evaluated for markers of collagen (picrosirius red), myofibroblast cells (α-SMA), and T lymphocyte infiltration (CD4 and CD8). Significantly more collagen was detected in MT-5 (Trp53 hotspot) orthotopic tumors, as compared with tumors from mice bearing KPC-Luc (Trp53 null) tumors (figure 2B,C). α-SMA was more prominent in MT-5 as compared with KPC-Luc tumors, although KPC-GEMM-derived tumors had greater α-SMA staining relative to the orthotopic models (figure 2D,E). Stromas in MT-5 tumors more closely mirrored that of KPC-GEMM-derived tumors (figure 2B–E). An activated stroma index (ASI) (ratio of myofibroblast over collagen deposit) exemplified this pattern, as KPC-Luc tumors had a high ASI compared with either MT-5 or KPC-GEMM tumors (figure 2F). An inverse relationship between these prominent stromal features and immune infiltrates was also evident, including significantly elevated CD4+ and CD8+ lymphocytes (figure 2G) in KPC-Luc tumors compared with MT-5 and KPC-GEMM tumors. In line with this IHC finding, flow cytometry analysis corroborated that KPC-Luc tumors contained more CD45+ cells (figure 2H) as well as more lymphocytic (CD3+) and granulocytic (Ly6G+) populations (figure 2I) than MT-5 tumors. Despite lower levels of total Ly6G+ cells (figure 2I), further evaluation of the Ly6G+ specific compartment (Ly6G+CD11c+Ly6C+) showed that KPC-Luc tumors contained more dendritic cells (DCs; characterized by CD11c+MHCII+Ly6C+), monocytess (characterized by Ly6C+CD11b+), and monocytic myeloid-derived suppressor cells (characterized by Ly6C+CD11b+) (figure 2J). In contrast, MT-5 tumors were populated with few monocytess, tumor-associated macrophages (TAMs; characterized by CD11b+Ly6C+CD206+), and DCs. Immunosuppressive cell surface receptors LAG3 and B7H4 were heightened on monocytess in KPC-Luc tumors compared with those found within MT-5 tumors, whereas no difference was found on Ly6G+ cells, DCs, or TAMs (online supplemental figure 1B).

To facilitate longitudinal imaging for in vivo studies, the stroma-rich MT-5 cell line was transduced to stably express luciferase (MT-5-Luc). This was achieved by isolating single-cell colonies of MT-5-Luc to select for pure clonal populations that expressed the most luciferase but did not mediate immunological rejection in animals on orthotopic pancreatic implantation (online supplemental figure 2A,B). Further, IHC analysis of α-SMA+ myofibroblasts, collagen, and CD4+CD8+ lymphocyte infiltration indicated orthotopic MT-5-Luc tumors phenocopied that phenotype of parental MT-5 tumors (online supplemental figure 2C,D).

We next explored the relationship between TP53 alteration and stromal gene signatures in human PDAC tumors using publicly available data from TCGA program. We hypothesized human PDAC tumors with hotspot TP53 mutations would show differential gene signatures of desmoplasia, as compared with those with wild-type or LOF TP53. As the PDAC data set inherently contained very few patients with hotspot TP53 mutations, we expanded our evaluation by including LUAD and LUSC, two cancer types driven by similar oncogenic signatures. Genomic data sets were organized according to TP53 mutational status (R172H, LOF, or wild-type) and assessed for signatures of normal or activated stroma, based on a previously published gene signature (online supplemental figure 2E). Expression of stromal genes in human samples suggests (with limited statistical power, due to limited cases with hotspot mutations) a decrease in normal stroma (p=0.05 for PDAC, p=0.90 for LUAD, p=0.02 for LUSC), with a corresponding more activated stroma (p=0.96 for PDAC, p=0.86 for LUAD, p=0.23 for LUSC) (online supplemental figure 2F–H). With a limited number of cases with hotspot mutations available in TCGA, the trends lack power but anecdotally support the observed trend in our mouse models (online supplemental figure 2F).

The desmoplasia of MT-5 tumors attenuates infiltration of mesoCAR T cells

We next tested our idea that tumors with more desmoplastic stroma blunt the ability of adoptively transferred T cells to infiltrate pancreatic malignancies. Past ACT approaches have evaluated the impact of stromal interference in PDAC mouse models by administering agents that interfere with the integrity of the stroma or its associated components. Yet studies on the efficacy of ACT across PDAC mouse models with varying levels of inherent stromal complexity are lacking. To address this idea in a fully immune-competent system, bulk CD3+ murine T cells were engineered with a CAR directed against murine mesothelin (mesoCAR T cells) that signals CD3ζ and 41BB on antigen recognition (online supplemental figure 3A). The expression of this protein as an antigenic target was validated on both KPC-Luc and MT-5 murine PDAC cell lines. Immunoblot analysis was used to assess the presence of mesothelin as both a precursor and a cleaved product (online supplemental figure 3B). Because CAR T cells are most adept at targeting cell surface antigens, we further verified whether mesothelin was detectable on cell surface levels via flow cytometry by comparing protein levels with an unstained control (online supplemental figure 3C). To address the relationships between stroma and T cell trafficking, mesoCAR T cells were transferred to mice bearing bioluminescence imaged and verified, orthotopic
Figure 2  Orthotopic MT-5 PDAC tumors parallel the immune landscape of KPC-GEMM tumors. (A) Whole tissue (left) and 20× magnification (right) of H&E-stained FFPE KPC-GEMM (left), KPC-Luc (middle), and MT-5 (right) tumor sections harvested at endpoint. (B) Picrosirius red and (C) α-SMA immunohistochemistry staining of KPC-GEMM (left), KPC-Luc (middle), and MT-5 (right) FFPE tumor sections. Quantification of stromal fibrosis by picrosirius red (D) and myofibroblastic positive stain by α-SMA (E) as measured by per cent positive cells of the total tumor area. The activated stroma index (F) was calculated by dividing the values of the α-SMA quantification by the picrosirius red values. (G) Representative images of CD4+ (red) and CD8+ (green) T cell immunofluorescence on whole tissue sections of KPC-GEMM, KPC-Luc, and MT-5 tumors. Quantification of CD4+ or CD8+ T cell infiltration (below) (% positive cells out of the total cell count in the tumor area). Percentage of CD45+ cells (H), percentage of CD3+, Ly6G−CD11b+, and Ly6C− out of the total CD45+ cells (I), and quantification of dendritic cells (DCs), monocytes, tumor-associated macrophages (TAMs), and monocyte myeloid-derived suppressor cells (M-MDSCs) out of the total Ly6C+ population (J) from KPC-Luc and MT-5 tumors by flow cytometry. Kruskal-Wallis (D, E, F, G, H) or one-way ANOVA (J) test: *p<0.05, **p<0.01, ***p<0.001. α-SMA, alpha-smooth muscle actin; ANOVA, analysis of variance; FFPE, formalin-fixed paraffin-embedded; GEMM, genetically engineered mouse model; PDAC, pancreatic ductal adenocarcinoma.
KPC-Luc (stroma-poor, *Trp53* null) or MT-5-Luc (stroma-rich, *Trp53* hot spot) tumors. All mesoCAR T cells were administered 10 days following implantation and 1 day following TBI as a lymphodepletion strategy (figure 3A). On day 7, following ACT, the mice were euthanized, and analysis of mesoCAR infiltrating lymphocytes by flow cytometry revealed significantly fewer present in stroma-rich MT-5-Luc tumors, as compared with stroma-poor KPC-Luc tumors (figure 3B and online supplemental figure 3D). In contrast, mesoCAR T cells were readily detectable in the blood of mice from both tumor models at equivalent levels (figure 3C). Importantly, the
abundance of donor mesoCAR T cells was validated in each tumor model via IHC staining for Thy1.1 protein levels (figure 3D, black arrows)—a congenic marker found only on the donor mesoCAR T cells—and the degree of desmoplasia at this time point was confirmed via IHC staining for picrosirius red (figure 3E).39

DISCUSSION

The stroma of PDAC continues to be a formidable challenge for the immune system to overcome with the currently available cancer treatment modalities, highlighting the urgent need for new therapeutic avenues.4,17

Previously published studies have attempted to enzymatically remodel the TME in combination with standard chemotherapy,46 but this approach did not improve objective tumor responses or overall survival. Current research suggests that higher quantities of tumor infiltrating lymphocytes correlate with improved overall survival and progression-free survival in patients with PDAC.39,49,50 Therefore, ongoing efforts continue to focus on novel strategies to target components of the TME that increase lymphocyte infiltration. To do so, a representative model system that recapitulates the human malignancy and its abundant desmoplasia is vital. In many ways, GEMMs can parallel human PDAC by using similar oncogenic drivers and mutations in relevant tumor suppressors. GEMMs bearing these mutations develop spontaneous PDAC tumors that follow a similar path to malignant progression as human patients. Thus, GEMM tumors are often considered the “gold standard” for modeling tumor composition in vivo.41 Unfortunately, GEMMs are highly variable, expensive, and time-consuming, creating significant drawbacks in their use for large animal studies. Due to the central role of the TME in PDAC progression and treatment outcomes, accurately modeling the microenvironment is crucial to reducing the disparity between preclinical data and human outcomes. By using immunocompetent, orthotopic tumor mouse models, our experiments have the dual benefit of enabling donor–host immune interactions, as well as tissue site-specific tumor pathology that recapitulates the TME of a human patient with PDAC. While human xenograft models lack reliable immune features and subcutaneous tumors lack site-specific physiology, our orthotopic model accommodates the best of both of these limitations.

Our data highlight both the utility and the limitations of distinct, immune-competent, orthotopic murine PDAC models for addressing hypotheses related to infiltration of therapeutic T cells. These results demonstrate unique phenotypic distinctions of the PDAC TME that occur despite seemingly similar genetic driver mutations in the tumor cells. In spite of the limited number of accessible human PDAC patient samples, our results suggest that Trp53 mutational burden deserves further investigation as a driver of desmoplasia in humans. Previous studies show that distinct Trp53 mutations can drive differences in macrophage recruitment and lymphocytic populations of the TME.32,44 Yet specific differences in desmoplasia in relation to mutational Trp53 burden in PDAC have not been well documented and negatively correlate with infiltration of adoptively transferred CAR T cells to these tumors. In alignment with other reports,45 these results support a role for desmoplastic stroma in disrupting access of tumor antigen-directed T cells into the TME and emphasize the importance of using realistic preclinical in vivo models to approximate the clinical features of patient PDAC tumors.

Mesothelin is overexpressed in human PDAC tumors and previous studies have validated its potential as a therapeutic target.39,40 While targeting mesothelin offers a promising avenue for treating PDAC, evidence suggests it will not be sufficient on its own. Within the PDAC TME, collagens are the most abundant ECM proteins deposited by CAFs and contribute to the process of desmoplasia that is driven by several mechanisms, including soluble factors such as transforming growth factor-β (TGFβ) and c-x-c motif chemokine ligand 10 (CXCL10).47 A large body of literature describes the role of desmoplastic stroma in restricting drug and lymphocyte infiltration into PDAC tumors.17,48–50 Studies modifying the levels of TME collagen, by depleting subsets of CAFs, accelerated tumor growth and reduced animal survival.57 However, collagen within the TME, which frames and anchors tumor tissue structure, can also stall PDAC progression by reducing cancer spread and promoting CD8+ T cell infiltration.51 Immunosuppressive functions elicited by CAFs through the secretion of cytokines IL-6 and CXCL1239,52 signify that CAFs are positioned in a unique, multifaceted role within the PDAC TME. By remodeling the PDAC stroma, preclinical studies indicate increased T cell infiltration and improved tumor control can be achieved through the use of TGFβ inhibitors in combination with ACT, or by targeting both stromal and tumor cells via a bispecific T cell engager.53,54 These data highlight a complex relationship between stroma composition and immune response in accordance with tumor development and suggest the need for multimodal therapies to fine-tune the PDAC TME.

Our results support the notion that access of lymphocytes to PDAC tumors is limited by the cellular components of the TME and potentially the nature of mutations driving the malignancy.54 By characterizing the differing stromal features of distinct orthotopic tumors, we can better adapt our preclinical testing of cellular or other immunotherapy approaches to maximize clinical relevance and applicability.

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