Development of an Orthotopic Model of Invasive Pancreatic Cancer in an Immunocompetent Murine Host

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Abstract

Purpose: The most common preclinical models of pancreatic adenocarcinoma utilize human cells or tissues that are xenografted into immunodeficient hosts. Several immunocompetent, genetically engineered mouse models of pancreatic cancer exist; however, tumor latency and disease progression in these models are highly variable. We sought to develop an immunocompetent, orthotopic mouse model of pancreatic cancer with rapid and predictable growth kinetics.

Experimental Design: Cell lines with epithelial morphology were derived from liver metastases obtained from KrasG12D+;LSL-Trp53R172H+;Pdx-1-Cre mice. Tumor cells were implanted in the pancreas of immunocompetent, histocompatible B6/129 mice, and the mice were monitored for disease progression. Relevant tissues were harvested for histologic, genomic, and immunophenotypic analysis.

Results: All mice developed pancreatic tumors by two weeks. Invasive disease and liver metastases were noted by six to eight weeks. Histologic examination of tumors showed cytokeratin-19–positive adenocarcinoma with regions of desmoplasia. Genomic analysis revealed broad chromosomal changes along with focal gains and losses. Pancreatic tumors were infiltrated with dendritic cells, myeloid-derived suppressor cells, macrophages, and T lymphocytes. Survival was decreased in RAG−/− mice, which are deficient in T cells, suggesting that an adaptive immune response alters the course of disease in wild-type mice.

Conclusions: We have developed a rapid, predictable orthotopic model of pancreatic adenocarcinoma in immunocompetent mice that mimics human pancreatic cancer with regard to genetic mutations, histologic appearance, and pattern of disease progression. This model highlights both the complexity and relevance of the immune response to invasive pancreatic cancer and may be useful for the preclinical evaluation of new therapeutic agents.

Ductal adenocarcinoma of the pancreas has the worst prognosis of all common epithelial malignancies, with an overall 5-year survival rate of <5% (1). Surgical resection offers the only hope for cure; however, <20% of patients are eligible for surgery and most patients eventually develop recurrence following resection (2). Gemcitabine and, more recently, erlotinib, an epidermal growth factor receptor tyrosine kinase inhibitor, are the only Food and Drug Administration–approved agents used in the treatment of advanced pancreatic cancer. Unfortunately, these therapies typically prolong survival by only a few months, and all patients ultimately succumb to progressive disease (2). The availability of appropriate animal models is therefore essential for the preclinical evaluation of novel therapeutic agents.

Most preclinical mouse models of pancreatic adenocarcinoma utilize immunodeficient hosts into which resected human tissues or established human cell lines (e.g., MiaPaCa2, PANC-1, BxPC3) are xenografted (3, 4). A major drawback of these models is the lack of an intact immune system in the host animal, which alters the tumor microenvironment and creates potentially significant differences from the human disease. This may in part explain the poor track record of immunodeficient models in predicting response to therapy in patients with pancreatic cancer (2). Immune cells that infiltrate tumors significantly impact the course of tumor growth and progression (5). Infiltrating immune cells have the potential to mount an effector response to limit tumor growth, but this response is often overcome by a combination of multiple factors, including the release of immunosuppressive cytokines by the tumor (e.g., interleukin-10) and the recruitment of immunosuppressive cell types (e.g., Gr-1+, CD11b+ myeloid-derived suppressor cells, tumor-associated macrophages, and FoxP3+ regulatory T cells; ref. 5). Immune cells can
also influence the formation and maintenance of tumor-supporting stroma, which in the case of pancreatic cancer can make up the bulk of the tumor and affect tumor resistance to chemotherapy and radiation therapy (6). Immunocompetent rat and hamster models of pancreatic cancer have been reported (7, 8); however, compared with the mouse, few reagents are available for identifying and isolating immune cells from these species.

In 2003, Hingorani et al. described a genetically engineered mouse model of pancreatic cancer in which expression of oncogenic Kras-G12D was limited to pancreatic tissue (9). The authors utilized a pancreas-specific promoter (Pdx-1) to drive expression of Cre-recombinase, which recognizes and excises a Lox-flanked stop repressor element upstream of the mutant Kras-G12D allele. As a result, these mice express mutant Kras-G12D driven by its native promoter resulting in the gradual development of pancreatic intraepithelial neoplasia (PanIN) that slowly progresses to invasive ductal adenocarcinoma (9). In 2005, the authors reported that progression to pancreatic cancer could be accelerated in mice by using the same Pdx-1-driven, Cre-Lox system to simultaneously induce expression of Kras-G12D and a mutant form of the p53 tumor suppressor gene (R172H; ref. 10). Of these mice, designated LSL-Kras-G12D/−;LSL-Trp53R172H/−;Pdx-1-Cre mice and cell lines were derived as previously described (10). Cells were grown in DMEM (high glucose 4.5 g/L) supplemented with 10% fetal bovine serum, 1 mol/L sodium pyruvate, 2 mmol/L L-glutamine, 1× nonessential amino acids (Gibco), and 1× penicillin/streptomycin (100 U/ml penicillin, 100 μg/ml streptomycin). Cells were plated at low density, specifically, ~50,000 cells/100-mm tissue culture dish (Fisher), in the above growth medium. Individual colonies displaying uniform epithelial morphology were cloned and subcultured. Individual colonies were then re-cloned for two more rounds. Purified cells (LM-P) were amplified in T75 flasks and vials of cells were stored at −125°C. In preparation for in vivo implantation, frozen cells were thawed and cultured in growth medium at 37°C in a humidified, 10% CO2 incubator. Subconfluent cells were detached with 0.25% trypsin and cell viability was assessed by trypan blue exclusion. LM-P cells were tested every six months for microbial contamination (including mycoplasma) using a PCR-based analysis and were found to be negative.

**Tumor implantation and monitoring**

To establish s.c. tumors, 10⁶ LM-P cells were resuspended in HBSS and injected under the skin overlying the right flank. To establish orthotopic tumors, mice were first anesthetized with a single i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), and then the pancreas was exposed through an abdominal incision (laparotomy). Two techniques were used for orthotopic tumor implantation: (a) direct injection of a single cell suspension of LM-P cells into the pancreas or (b) transplantation of a s.c. LM-P tumor fragment onto the pancreas. For direct injection, 10⁶ cells were resuspended in 50 μl HBSS and injected into the pancreas using a 30G needle (BD). Leakage occurred in ~5% of mice, and only mice without leakage during injection were included in experiments. For
s.c. tumor transplantation, 1-cm tumors were harvested and minced into 3- to 4-mm fragments. One tumor fragment was secured to the pancreas using 7-0 Prolene suture. After tumor implantation (with either technique), the pancreas was carefully returned to the peritoneal cavity and the abdomen was closed with 4-0 Vicryl suture. After tumor implantation, all mice were monitored at least twice weekly for disease progression by abdominal palpation and for overall signs of morbidity such as ruffled fur, hunched posture, and immobility. Moribund mice were euthanized by CO₂ inhalation. For survival studies, mice were followed until death or euthanized by a blinded observer when signs of morbidity were evident.

**Tumor progression**

A separate cohort of mice that had undergone either LM-P cell injection (n = 3) or LM-P tumor fragment transplantation (n = 3) underwent repeat laparotomy to directly measure tumor size at two, four, and six weeks after implantation. Tumor volume was calculated based on the formula for ellipsoid volume, \( V = \frac{4}{3} \pi \times \frac{a}{2} \times \frac{b}{2} \times \frac{c}{2} \) where a, b, and c represent the maximal length, width, and height, in millimeters, respectively.

**Tumor implantation using PDA cell lines**

PDA1-1 and PDA3-5 cells were derived from primary tumors found in the pancreas of KrasG12D/+.LSL-Trp53R172H/+. Pdx-1-Cre mice, using a similar protocol for isolation, purification, and microbial testing as described above for LM-P cells. Orthotopic tumor implantation into immunocompetent B6/129 mice was done, and disease progression and tumor histology were assessed.

**Histologic analysis**

Pancreatic tumors, livers, and lungs were harvested from mice at three and six weeks after orthotopic tumor implantation. Tissues were placed in 10% phosphate-buffered formalin and 24 to 72 hours later were embedded in paraffin. Sections (4-5 μm) were dewaxed in three changes of xylene and brought to water through graded alcohols. Representative sections were stained with H&E. For immunohistochemistry, sections were antigen-retrieved in rodent decloaker antigen retrieval solution (Biocare Medical) in a pressure cooker. Peroxidase activity was blocked using 3% hydrogen peroxide (Lab Vision). Sections were antigen-retrieved in rodent antimouse FoxP3 (APC) versus isotype control antibody, antimouse FoxP3 (APC) versus isotype control antibody, and processed into single cell suspensions by dissociation and mechanical dissociation (GenTeMACS Tissue Homogenizer, Miltenyi Biotec). Tumor genomic DNA was isolated using a Qiagen DNA/RNA Allprep Mini kit. Array comparative genomic hybridization (CGH) was done according to the manufacturer’s protocol on Agilent 44 K murine whole genome arrays. Briefly, 3 μg of genomic DNA from each test sample were random primer-labeled with Cy5 and cohybridized to the microarray along with 3 μg of Cy3-labeled normal pancreas reference DNA from syngeneic mice. Tuor/normal log₂ ratios were extracted using Agilent software (CGH Analytics), and gains and losses were called using the circular binary segmentation algorithm (11).

**Comparative genomic hybridization**

Tumor genomic DNA was isolated using a Qiagen DNA/RNA Allprep Mini kit. Array comparative genomic hybridization (CGH) was done according to the manufacturer’s protocol on Agilent 44 K murine whole genome arrays. Briefly, 3 μg of genomic DNA from each test sample were random primer-labeled with Cy5 and cohybridized to the microarray along with 3 μg of Cy3-labeled normal pancreas reference DNA from syngeneic mice. Tuor/normal log₂ ratios were extracted using Agilent software (CGH Analytics), and gains and losses were called using the circular binary segmentation algorithm (11).

**Immune cell isolation and surface marker staining**

Pancreatic tumors were minced using a sterile scalpel blade, and single cell suspensions were generated using a combination of enzymatic digestion (600 U/mL collagenase IV, Worthington) and mechanical dissociation (GenTeMACS Tissue Homogenizer, Miltenyi Biotec). Tumor debris was removed using 70- and 100-μm filters (BD Falcon). Perivascular draining lymph nodes were harvested and processed into single cell suspensions by dissociation over filters. Pancreas and draining lymph nodes from tumor-naive mice were used as controls for comparison with tissue obtained from tumor-bearing mice. Cells from all tissues were washed with PBS containing 2% fetal bovine serum, Fc blocked (1:50 dilution, BioLegend), and then stained using a cocktail of antimouse antibodies against CD45.2 (PacBlue or Alexa700); lineage markers CD19, NK1.1, TCR (PE); B220 (PetXR); CD11c (PECy7); CD11b (PacBlue); Gr-1 (APCCy7); I-Ab (APC); CD3 (FITC); CD4 (PECy7); CD8 (Alexa700). All antibodies were purchased from BD Biosciences or BioLegend. Isotype-matched antibodies were used as negative controls when appropriate. Surface marker expression was determined by multicolor flow cytometry on an LSR II flow cytometer, and data were analyzed using FlowJo 8.7.1 software.

**Intracellular staining for FoxP3**

Tumor-infiltrating T cells were identified with labeled antibodies to T-cell surface markers and then permeabilized and fixed using the Mouse Regulatory T Cell Staining Kit (eBioscience). Intracellular staining was done using antimouse FoxP3 (APC) versus isotype control antibody, as part of the Staining Kit.

**Survival of immunocompetent versus immunodeficient mice**

After establishing growth kinetics with 10⁸ and lower cell numbers, B6/129 (n = 6) and immunodeficient RAG−/− mice (n = 5) were orthotopically injected with 10⁸ LM-P cells and then monitored for survival.

**Treatment of LM-P tumors with gemcitabine and LY364947**

For evaluation of in vitro cytotoxicity, LM-P cells were first plated in quadruplicate (2.5 × 10⁴ cells/well) onto 96-well plates and allowed to grow for 24 hours. Escalating
concentrations of gemcitabine (courtesy of Clinical Pharmacy, Stanford Hospital) or a transforming growth factor (TGF-β) receptor-1 kinase inhibitor (LY364947, Calbiochem) were then added and MTT assay (R&D Systems) was done 48 hours later, as per the manufacturer’s protocol. For in vivo studies, orthotopic tumor implantation was done using later passaged (P8), more aggressive LM-P cells. At two weeks, mice with similarly sized pancreatic tumors (determined by palpation) were randomized to receive PBS, gemcitabine alone, or gemcitabine plus the TGF-β receptor-1 kinase inhibitor. Gemcitabine was given at 100 mg/kg by i.p. injection on days 0, 3, 6, and 9 after randomization. The TGF-β receptor-1 kinase inhibitor was given at 1 mg/kg i.p. on days 0, 2, 4, 6, and 8.

**Statistical analysis**

Tumor volume comparisons were analyzed using the paired Student’s t-test. The frequency of tumor uptake and metastases between groups was analyzed using Fischer’s exact test. Survival differences between groups were assessed with the log-rank test using GraphPad Prism 5.02 software. *P < 0.05* was considered statistically significant.

**Results**

**Pancreatic tumor uptake is uniform and disease progression occurs predictably**

After either (a) injection of suspended LM-P cells into the pancreas or (b) implantation of a s.c. LM-P tumor fragment onto the pancreas, tumors were noted as early as two weeks and progressed predictably by four weeks (Fig. 1A). Comparison of average tumor volumes at two and four weeks showed no statistically significant differences, suggesting similar growth kinetics with either technique (Fig. 1B). By six weeks, tumors in some mice became difficult to measure owing to their extensive, locally invasive nature and poorly defined borders (Fig. 1C, left). At this stage in disease progression, liver metastases were also frequently noted (Fig. 1C, right) and the mice appeared ill. Of note, some mice also developed biliary and gastric outlet obstruction during the course of disease progression (Fig. 1D). By eight weeks, mortality was consistently 100% (data not shown).

In total, pancreatic tumors developed in 100% of mice following orthotopic implantation of LM-P cells (Table 1). Of these mice, 90% developed liver metastases whereas no mice developed peritoneal carcinomatosis or hemorrhagic ascites. No significant differences were noted in the frequencies of uptake and metastases with either technique. Lung metastases were noted in some mice, particularly those with more advanced disease; however, the frequency of metastasis to this organ was not systematically evaluated. Pancreatic tumors and liver metastases also developed with <10⁶ cells injected, but tumor volumes were initially smaller and disease progression occurred more slowly; in contrast, the use of cells passaged more frequently *in vitro* generated pancreatic tumors with more rapid disease progression (data not shown).

**Pancreatic and metastatic tumor histology resembles human disease**

H&E staining of representative LM-P pancreatic tumors showed adenocarcinoma with high-grade nuclear features, areas of necrosis and hemorrhage, and regions of desmoplasia (Fig. 2A). Adjacent areas of poorly differentiated, solid sheets of cells were also noted, particularly in more advanced tumors. Cytokeratin-19, a marker expressed in most human pancreatic ductal adenocarcinomas, was expressed in tumors as shown by immunostaining (Fig. 2B). Glandular areas showed more consistent cytoplasmic CK-19 staining than poorly differentiated areas of the tumor. In many pancreatic tumors, perineural invasion was present (Fig. 2C) and peripancreatic lymph nodes were often heavily infiltrated by tumor (Fig. 2D). Histologic examination of liver and lung metastases (Fig. 2E and F, respectively) confirmed the presence of adenocarcinoma.

**Carcinomatosis and more aggressive disease progression occur when pancreatic tumors are generated from primary tumor cell lines**

Orthotopic implantation of PDA1-1 and PDA3-5 cell lines, which were derived from primary pancreatic tumors (as opposed to liver metastases for LM-P) in the Kras(gef129/+);LSL-Trp53R172H/+;Pdx-1-Cre mice, led to pancreatic tumor development in 100% of the mice (data not shown). Mice implanted with PDA1-1 cells had significantly worse survival compared with mice implanted with PDA3-5 or LM-P (Fig. 1E). In contrast to LM-P–implanted mice (Table 1), all of the PDA1-1– and PDA3-5–implanted mice developed extensive peritoneal carcinomatosis (Fig. 1F) and only occasionally, small liver metastases. On histologic analysis, PDA1-1 pancreatic tumors were poorly differentiated (Fig. 2G) compared with PDA3-5 (Fig. 2H) and LM-P (Fig. 2A), which correlated with the survival data.

**CGH on tumor cells from different sources**

To characterize our tumor model at the genomic level, we carried out array CGH on *in vitro* propagated LM-P cells, a s.c. tumor derived from LM-P cells, and an orthotopic tumor derived from a transplanted s.c. tumor (Fig. 3). The LM-P cells displayed several broad low-level gains and losses along with one focal deletion on cytoband 4D1. It is possible that these genomic aberrations might cooperate with the Kras and p53 mutations to transform cells. The s.c. tumor mirrored the LM-P cells with the exception of modest gains on chromosomes 5A1, 5G3, and 6A1. The orthotopic tumor genome shared some features with the LM-P cells and the s.c. tumor but also contained many new genomic aberrations, including further gains on the ends of chromosomes 5 and 6. Interestingly, among many other genes, hepatocyte growth factor and its receptor, Met, reside in these regions on chromosomes 5 and 6, respectively, which were further amplified with progression from *in vitro* cells to orthotopic tumors.
Fig. 1. Early and late disease in our orthotopic model. At 2 weeks, LM-P tumor uptake is noted, with progressive growth at 4 weeks. Comparison of the two techniques for implantation (see Materials and Methods) reveals no difference in tumor appearance (A) or total tumor volume (B) at these early time points. C, by 6 to 8 weeks, mice develop extensive local growth into adjacent organs (dotted line, tumor invading into stomach) and liver metastases (open arrows). D, in some mice, clinically relevant disease manifestations, such as biliary obstruction (arrowheads, dilated common bile duct) producing jaundice (swab tip of bilious peritoneal fluid), and gastric outlet obstruction (*, massively dilated stomach) are seen. In comparison with LM-P, orthotopic implantation of PDA cells results in more aggressive disease progression with decreased survival (for PDA1-1, E; *, P < 0.05) and development of carcinomatosis (for both PDA1-1 and PDA3-5, F).
Immunoophenotyping of tumor-infiltrating mononuclear cells reveals a complex immune response

Single cell suspensions obtained from processed LM-P pancreatic tumor and draining lymph nodes three weeks after orthotopic tumor implantation were analyzed by flow cytometry for surface marker expression. After gating in CD45+ hematopoietic cells and gating out lineage-positive (CD19, NK1.1, TCR) cells, multiple expanded populations of CD11c+ and CD11b-expressing cells were noted in pancreatic tumors compared with the pancreas of tumor-naive normal mice (Fig. 4A). Population 1 (CD11c-high, CD11b-negative) and population 2 (CD11c-high, CD11b-positive) were characterized as dendritic cells as both populations also expressed high levels of MHC Class II (I-A<sup>β</sup>). A small proportion of cells in these two dendritic cell populations also coexpressed CD86, an activation marker and T-cell costimulatory factor; however, the majority (80-90%) lacked CD86 expression. Population 3 (CD11c-negative/low, CD11b-positive) did not express either MHC Class II or CD86 (data not shown), but could be further subdivided into Gr-1–positive myeloid-derived suppressor cells (MDSC) and Gr-1–negative macrophages. Each of these three myeloid cell populations was also expanded, but to a lesser extent, in the adjacent tumor-draining lymph nodes. In both the pancreatic tumors and draining lymph nodes, over two thirds of the T cells (CD45+ CD3+) were CD4+ (Fig. 4B). A proportion (>20%) of these cells expressed CD25 and intracellular FoxP3, the phenotype of regulatory T cells. A similar immune cell infiltrate consisting of dendritic cells, MDSCs, macrophages, and regulatory T cells was noted in pancreatic tumors and draining lymph nodes as early as 1.5 weeks after orthotopic tumor implantation (data not shown). When compared with normal control tissues, no consistent changes in natural killer (NK) or B-cell populations were found in either tumor or lymph nodes at 1.5 or 3 weeks (data not shown).

Impact of an adaptive immune response on survival

When LM-P tumor cells were orthotopically implanted into RAG−/− mice, which lack T and B cells, these mice showed more rapid disease progression and, overall, significantly decreased survival compared with fully immunocompetent B6/129 mice (Fig. 4C).

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Effect of gemcitabine and TGF-β receptor-1 kinase inhibition on LM-P tumor growth

To evaluate the sensitivity of LM-P tumors to an agent with known activity against human pancreatic cancer, we cultured these cells in the presence of gemcitabine. We also studied the effect of a TGF-β1 receptor-1 kinase inhibitor, LY364947, based on recent studies indicating a role for TGF-β in the growth of pancreatic tumors and development of metastases (12, 13). In vitro, gemcitabine resulted in significant cytotoxicity against LM-P cells at all concentrations tested. In contrast, some cytotoxicity was noted with LY364947 only at higher concentrations tested (Fig. 5A). In vivo, gemcitabine alone significantly improved survival in mice with pancreatic tumors, an effect that was slightly enhanced by coadministration of LY364947 (Fig. 5B).

Discussion

The development of a genetically engineered mouse model of pancreatic cancer marks a significant turning point in preclinical model development in pancreatic cancer (14). Other groups have shown that mutant Kras in combination with other targeted genetic mutations, including Ink4a/Arf and MUC1, can also accelerate the development and progression of pancreatic cancer (15, 16). These spontaneous mouse models of pancreatic cancer in immunocompetent mice have significant advantages over traditional, immunodeficient xenograft models, in which the host environment and the local tumor microenvironment bear little resemblance to the natural milieu in which tumors typically develop and metastasize in humans.

Although our orthotopic implantation model is not a spontaneous model, it has several advantages over genetically engineered models. The most obvious advantage is that the model is rapidly established without the need for time-consuming and expensive breeding. Furthermore, the disease develops rapidly and predictably. In LSL-Kras<sup>G12D</sup>;<LSL-Trp53<sup>R172H</sup>;<Pdx-1-Cre mice, disease latency is highly variable, ranging from weeks to almost one year of age (10). With either direct tumor cell injection or s.c. tumor fragment transplantation, all mice in our study developed palpable pancreatic tumors by two weeks that progressed to advanced disease by six to eight weeks (Fig. 1). The rapidity and consistency of disease development in our model makes it possible to carry out...
Fig. 2. Histology of pancreatic tumors and metastases. A. LM-P tumors form glands and regions of desmoplasia (arrows). B, immunohistochemistry shows that pancreatic tumors stain for cytokeratin-19, a ductal epithelial marker (brown, cytokeratin-19; blue, hematoxylin counterstain). Areas of perineural invasion (C; arrowheads, nerve; dotted line, tumor invasion) and peripancreatic lymph node infiltration (D; T, tumor; GC, germinal center) are also noted. Liver (E) and lung (F) metastases confirm adenocarcinoma. PDA pancreatic tumors show similar histology to LM-P but are predominantly poorly (1-1; G) or well differentiated (3-5; H). H&E, original magnification, × 100 for A and D to F, × 200 for B, C, G and H.
time-dependent and large-scale studies in a resource- and cost-effective manner.

Our orthotopic mouse model of pancreatic cancer is established by surgical implantation of tumor cells into the pancreas of an immunocompetent host. Although other cell line–based immunocompetent mouse models of pancreatic cancer exist (17, 18), the cells used in those models are toxic and virally induced. Close examination of reports using these models reveals that the tumor histology is predominantly sarcomatoid, with tumors mainly composed of solid sheets of cells (19). In contrast, the cells used to establish our model were “induced” by mutations in Kras and p53, mutations found in over 90% and 60% of human pancreatic cancers, respectively (2). Histologic examination of the tumors in our model showed regions of clear glandular differentiation and some regions of desmoplasia (Fig. 2A), which are characteristic of human pancreatic cancer. Moreover, pancreatic tumors in our model expressed cytokeratin-19 (Fig. 2B), a marker expressed by most human pancreatic ductal adenocarcinomas. Recently, Olive et al. also reported on the use of a mouse pancreatic cancer model generated by implantation of tumor cell lines derived from LSL-KrasG12D/+;LSL-Trp53R172H/+;Pdx-1-Cre mice (20); however, most mice had s.c. tumors, and only human cell lines (e.g., MiaPaCa2) were used to establish orthotopic xenograft tumors.

With regard to initial disease development, when LSL-KrasG12D/+;LSL-Trp53R172H/+;Pdx-1-Cre mice develop tumors, they often occur throughout the pancreas, as all ductal epithelial cells within the organ harbor Kras and p53 mutations and are susceptible to malignant transformation (10). In contrast, in orthotopic implantation models such as ours, the host pancreas cells are wild type and tumors eventually invade into this otherwise normal parenchyma, similar to what occurs in human pancreatic cancer. A disadvantage of orthotopic implantation, however, is that tumor development does not proceed through the typical stepwise progression of preinvasive stages (PanIN); thus, carcinogenesis may be better studied using the original LSL-KrasG12D/+;LSL-Trp53R172H/+;Pdx-1-Cre mice.

We chose cells derived from liver metastases found in the LSL-KrasG12D/+;LSL-Trp53R172H/+;Pdx-1-Cre mice based on the rationale that cells that have already metastasized to the liver have a higher propensity to metastasize again, as shown in other mouse models of cancer (21, 22). Indeed, 90% of our mice implanted with LM-P cells developed liver metastases (Table 1). In contrast, we observed that mice with pancreatic tumors generated from PDA (primary tumor) cell lines had fewer liver metastases and uniformly developed carcinomatosis. Our findings suggest that, depending on which cell line is used, different aspects of pancreatic cancer disease progression may be highlighted for closer study, with the advantage of more rapid and predictable kinetics compared with the original genetically engineered mice.

The genome of human pancreatic cancer exhibits substantial complexity, and CGH array analysis of the pancreatic tumors in our model indicates similar complexity. In addition to Kras and p53, it is well accepted that many more genetic alterations are involved in pancreatic carcinogenesis (23). Additionally, these genomic abnormalities increase with disease progression (23). This progressive genomic instability is mimicked in our model as revealed by CGH data obtained from in vitro cultured cells, s.c. tumors, and invasive pancreatic tumors (Fig. 3). The data point to interesting genomic regions that may harbor genes involved in disease progression; however, due to the complex nature of the genome of pancreatic cancer, analysis of additional samples together with mechanistic studies on candidate genes will be required to confirm the identities and roles of involved genes.

Characterization of the immune cells found within the tumors and draining lymph nodes in our model revealed that the immune response to invasive pancreatic cancer is highly complex and involves an infiltration of tumors by multiple immune cell types (Fig. 4A, B). Some populations, such as MDSCs, tumor-associated macrophages, and regulatory T cells, are known to inhibit effector T cell and NK activity, allowing for tumor escape (5, 24, 25). In contrast, dendritic cells, defined by their high expression of MHC Class II and activation markers/T-cell costimulatory
molecules (e.g., CD86), are the most potent antigen-presenting cells and are capable of orchestrating an adaptive antitumor immune response (26, 27). However, poorly activated “tolerogenic” dendritic cells have been shown to induce T-cell anergy, allowing for continued tumor growth and progression (28, 29). Immunophenotypic analysis of the tumor-infiltrating dendritic cells in our model would suggest that, within the tumor

**Fig. 4.** LM-P tumors are infiltrated with distinct mononuclear cell types. A, three expanded populations are seen in pancreatic tumors and, to a lesser extent, in draining lymph nodes (dLN), based on surface marker expression: population 1, CD11c-high, CD11b-negative; population 2, CD11c-high, CD11b-positive; and population 3, CD11c-negative/low, CD11b-positive cells. The two CD11c-high populations (1 and 2) express MHC Class II, and a small proportion of the cells also express CD86. The CD11c-negative/low CD11b-positive cells (population 3) are composed of Gr-1+ MDSCs and Gr-1− tumor-associated macrophages. B, the T-cell response in pancreatic tumors and draining lymph nodes is shifted toward CD4+ cells. Tumor-infiltrating CD4+ T cells (+) include a distinct population of CD25+ FoxP3+ regulatory T cells. Numerical values represent CD4:CD8 ratios. Isotype controls (for CD86, FoxP3) are shown in gray. C, lymphocyte-deficient RAG−/− pancreatic tumor-bearing mice have decreased survival compared with fully immunocompetent mice (*, P < 0.05); however, both ultimately succumb to disease.
microenvironment, the majority of dendritic cells are tolerogenic; however, a small proportion of the dendritic cells present may be appropriately activated, based on CD86 expression (Fig. 4A).

The results from the survival study (Fig. 4C) suggest that, in the fully immunocompetent B6/129 mice, at least some degree of effective dendritic cell priming of T cells against tumor likely occurs, leading to improved survival compared with T lymphocyte–deficient RAG−/− mice. This is supported by our finding of activated dendritic cells in the tumor-draining lymph nodes (Fig. 4A and data not shown), which suggests that at least some of these cells successfully trafficked to sites where priming of naïve T cells can occur. In time, however, all mice succumbed to progressive disease, indicating that the adaptive immune response is ultimately ineffective, perhaps thwarted by the simultaneous presence of tolerogenic dendritic cells, MDSCs, macrophages, and regulatory T cells. In fact, these particular cell types have been identified as key elements hindering the efficacy of vaccine-based immunotherapy for human pancreatic cancer (30).

Clark et al. analyzed the immune response in KrasG12D-expressing, p53 wild-type mice, which manifest precursor PanIN that occasionally progresses to invasive adenocarcinoma (31). They found, surprisingly, that MDSCs and regulatory T cells were already present in the early PanIN stages and continued to increase with disease progression (31). The dendritic cell response was not analyzed; however, the authors did note that about one third of intratumor CD4+ T cells had an expression profile (CD45RB-low and CD44-high) that suggests evidence of dendritic cell priming and antigen experience (31). In another spontaneous, genetically engineered mouse model of pancreatic cancer (TGF-α/p53 mutant), tumor-specific CD8+ T cells were noted (32); however, the presence of activated dendritic cells, which would likely contribute to this response, was also not assessed.

To further assess the similarity of our model to human pancreatic cancer, we evaluated the sensitivity of LM-P tumors to gemcitabine, a chemotherapeutic agent widely used in the treatment of human pancreatic cancer (2). We also evaluated the effect of LY364947, a novel TGF-β receptor-1 kinase inhibitor. TGF-β is one of several tumor-derived cytokines that are thought to induce extracellular matrix deposition and fibrosis, creating the desmoplastic stroma characteristic of pancreatic cancer (6). Also, inhibition of TGF-β signaling has been shown in a s.c. xenograft (BxPC3) mouse model of pancreatic cancer to potentiate drug delivery by altering permeability at the tumor microenvironment level (12). Whereas gemcitabine was highly cytotoxic to LM-P cells in vitro and extended survival of tumor-bearing mice in our orthotopic, immunocompetent

![Figure 5](https://example.com/figure5.png)

**Fig. 5.** Treatment of LM-P tumors with gemcitabine and LY364947, a TGF-β receptor-1 kinase inhibitor (TGF-βR1 KI). A, in vitro, MTT assays show direct cytotoxicity of gemcitabine but not TGF-βR1 KI against LM-P cells. B, in vivo, gemcitabine treatment results in improved survival, an effect slightly enhanced by addition of TGF-βR1 KI. Gem, gemcitabine; *, P < 0.05 for Gem versus PBS control; **, P < 0.05 for Gem + TGF-βR1 KI versus Gem alone.)
model, LY364947 had little or no antitumor activity as a single agent in vitro but resulted in modest improvement in survival when used in combination with gemcitabine (Fig. 5). These results are consistent with the concept that TGF-β inhibition likely acts on surrounding tumor stroma. It will be interesting to determine if optimizing the dose of this agent results in greater efficacy.

Our preliminary findings with gemcitabine and TGF-β inhibition, however, should be approached with some caution. Olive et al. showed recently that due to differences in tumor-supporting stroma and microvasculature, transplant models of pancreatic cancer were more similar to gemcitabine than the original genetically engineered mice (20). Although desmoplasia was noted in pancreatic tumors in our model (Fig. 2A), the extent and pattern of fibrosis may not be as profound as in the genetically engineered mice or in humans. Our results with gemcitabine and TGF-β inhibition will likely need to be further validated in the original genetically engineered mice; nonetheless, we envision that our model can serve as a complementary resource and cost-effective screening tool for preclinical testing of new therapeutic agents.

In conclusion, we have developed a rapid, predictable model of pancreatic cancer by orthotopic implantation of tumor cells into immunocompetent mice. Like the human disease, tumor cells have both Kras and p53 mutations, and the tumors that develop from these cells have a histologic appearance and pattern of disease progression similar to the human disease. Genomic analysis suggests that this model may help to reveal new genes involved in disease progression. Immunophenotypic analysis highlights the complexity of the immune response to pancreatic cancer. Our hope is that this model will further the understanding of pancreatic cancer biology and prove useful for the preclinical evaluation of new therapeutic agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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