Chemo-immunotherapy mediates durable cure of orthotopic Kras^{G12D/p53−/−} pancreatic ductal adenocarcinoma

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ABSTRACT
Pancreatic ductal adenocarcinoma (PDAC) is the third leading cause of cancer-related death in the United States, exhibiting a five-year overall survival (OS) of only 7% despite aggressive standard of care. Recent advances in immunotherapy suggest potential application of immune-based treatment approaches to PDAC. To explore this concept further, we treated orthotopically established K-rasG12D/p53−/− PDAC tumors with gemcitabine and a cell-based vaccine previously shown to generate durable cell-mediated (T_{H1}) immunity. Tumor progression was monitored by IVIS. The results indicated that the combination of chemotherapy and dendritic cell (DC) vaccination was effective in eliminating tumor, preventing metastasis and recurrence, and significantly enhancing OS. No animal that received the combination therapy relapsed, while mice that received gemcitabine-only or vaccine-only regimens relapsed and progressed. Analysis of circulating PBMC demonstrated that mice receiving the combination therapy exhibited significantly elevated levels of CD8^{+} IFNγ−/− CCR7−/− NK1.1^{+} T-cells with significantly reduced levels of exhausted GITR^{+} CD8^{+} T-cells after the cessation of treatment. Retro-orbital tumor re-challenge of surviving animals at six-months post-treatment demonstrated durable antitumor immunity only among mice that had received the combination therapy. CD8^{+} splenocytes derived from surviving mice that had received the combination therapy were sorted into NK1.1^{+} and NK1.1^{−} populations and adoptively transferred into naive recipients. Transfer of only 1,500 CD8^{+} NK1.1^{+} T-cells was sufficient to mediate tumor rejection whereas transfer of 1,500 CD8^{+} NK1.1^{−} T-cells imparted only minimal effects. The data suggest that addition of a T_{H1} DC vaccine regimen as an adjuvant to existing therapies can mediate eradication of tumors and offer durable protection against PDAC.

Introduction
Pancreatic ductal adenocarcinoma (PDAC) is the tenth most common cancer worldwide, yet the third most common cause of neoplastic death in the United States. Modern chemotherapeutic regimens including FOLFIRINOX (folinic acid, 5'-fluorouracil, irinotecan, oxaliplatin) and gemcitabine/nab-paclitaxel can increase median survival to 11 mo in selected patients with good performance status. Pancreatectomy with curative intent is possible in fewer than 20% of cases and increases median survival to only 20–23 mo in conjunction with adjuvant or neoadjuvant therapy; five-year overall survival (OS) remains a stubborn 7%. In the United States in 2016, there will be an estimated 53,000 pancreatic cancer diagnoses (all types) and nearly 42,000 deaths. Every year since 2000, the incidence rate of PDAC has increased by 1.2%, and the death rate by 0.4%. Given these grim statistics, it is clear that there remains an unmet medical need for better treatment regimens that can enhance survival in PDAC.

The use of immunotherapy for the treatment of PDAC is an area of active investigation, and attempts at targeting PDAC by vaccine immunotherapy have shown some success. Of note, Lepisto et al. reported 33% four-year survival among 12 PDAC patients treated with MUC1 peptide-loaded dendritic cells (DC). And Morse et al. reported that 3 of 3 PDAC patients treated with CEA mRNA-transfected DC were alive with NED at 30 mo post-vaccination. Additionally, several animal models have also established the utility of using total tumor antigens (mRNA or lysate) for DC immunotherapy, a critical point given the significant contribution of desmoplasia to the total antigenic content of the pancreatic tumor. Old vaccination concepts for PDAC have also been given new life with the advent of immune checkpoint inhibitor drugs. In a recent randomized trial in which the anti-CTLA-4 inhibitor drug ipilimumab was compared to ipilimumab + GVAX (allogeneic irradiated cancer cells transduced with GM-CSF) in heavily pretreated patients with advanced PDAC, patients assigned to the ipilimumab + GVAX arm exhibited significantly greater median OS (5.7 mo vs. 3.6 mo) and 1 y OS (27% vs. 7%). Most importantly, 2 of 15 patients in the ipilimumab + GVAX arm experienced...
long-term disease stabilization and were alive at 30 mo whereas all 15 patients receiving ipilimumab-only had died by 17 mo post-treatment. In addition, investigators have demonstrated that effective anticancer immune responses can specifically target the neoplastic support stroma of a variety of different tumor types. Hence the presence of desmoplasia commonly associated with PDAC does not appear to be a barrier to immunotherapy.

DC are the most important of the professional antigen presenting cells (APCs) that initiate and direct adaptive immune responses. Upon detection of a danger signal DC migrate to local lymph nodes where they engage T cells and, depending upon a broad host of variables, induce a variety of immunogenic or tolerogenic responses. For the past decade our group has been interrogating a basic regulatory mechanism that adds to the paradigm by which regulation of cellular immunity is understood. We have previously demonstrated that the loading of DC with antigenically similar (homologous) MHC class I and II antigens leads to a cell-intrinsic upregulation of $T_{H1}$ polarization. This polarization consists of a differential ability to secrete IL-12, IL-23, CTLA-4,11 and the novel $T_{H1}$-polarizing cytokine AIMp1,12-15 to upregulate surface CD83 and CD40, and to induce the preferential generation of IFN-$\gamma$-secreting CD8$^+$ cytolytic T-cells. DC loaded in this manner also demonstrate a substantial reorganization of the transcriptome, exhibiting a transcriptional profile significantly different from alternatively loaded DC by over 1,700 transcripts in areas such as interferon signaling, antigen-presentation, antiviral responses, protein ubiquitination, and DC licensing. Using nearly 40 different independent antigenic systems, previous work demonstrated that loading of DC with antigens of similar or identical content stimulates release of the $T_{H1}$-promoting cytokine AIMp1/p43 with concomitant reduction in the release of CTLA-4$^+$ regulatory microvesicles, and that these events are not dependent on innate pattern recognition or TLR ligands. This concept has subsequently been utilized for the generation of cell-based vaccine platforms that specifically target cancer with highly cytotoxic T-cell responses of durable memory potential. Here, we have generated such a platform for experimental treatment of orthotopically established K-ras$^{G12D}$/p53$^{-/-}$ PDAC tumors in mice and report that a combination therapy of $T_{H1}$ DC vaccination and standard of care gemcitabine results in durable elimination of tumor through the generation of long-lived memory cells that prevent relapse. Additional data suggest that antitumor immunity may be dependent upon a subpopulation of CD8$^+$NK1.1$^+$ T-cells not previously described in the context of cancer.

**Materials and methods**

**Reagents**

Antibodies: $\alpha$Human/mouse CTLA-4 (WB) (Abcam; Cambridge, MA); $\alpha$Human/mouse AIMP1 (Lifespan Biosciences Inc., Seattle, WA); $\alpha$Human/mouse $\beta$-actin was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Flow cytometry antibodies for staining against CD3, CD25, CD8, CD11c, CD80, CD83, CD86, IFN-$\gamma$, NK1.1, CCR7, Lag3, GITR, and CD152 were purchased from BD Biosciences (San Jose, CA).

Six to eight-week-old female C57BL/6 mice were obtained from Baylor College of Medicine’s Center for Comparative Medicine. All mice were maintained in accordance with the specific IACUC requirements of Baylor College of Medicine.

**Orthotopic tumor implantation**

Tumor cells were orthotopically implanted in mice. Briefly, mice were anesthetized with isoflurane and a 1 cm incision in the left subcostal region was made. Murine PDAC cells (Kras$^{G12D}$/p53$^{-/-}$/luc2$^+$) were injected into the caudal pancreas at 0.5–1.0 $\times 10^6$ cells per mouse. The peritoneum and skin were closed with the sutures. Buprenorphine (0.5 mg/kg) was administered postoperatively to minimize pain. Each experimental cohort consisted of five mice per group unless otherwise indicated. The Kras$^{G12D}$/p53$^{-/-}$/luc2$^+$ cell line was obtained from Dr. Steven Ullrich at the University of Texas MD Anderson Cancer Center in August, 2014. This cell line was authenticated by the University of Texas MD Anderson Cancer Center Characterized Cell Line Core in April, 2013 by short tandem repeat (STR) DNA fingerprinting using the AmpFISTR Identifiler Kit according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA).

**Dendritic cell manipulations**

DC were derived from the long bones of C57BL/6 mice. Bone marrow leukocytes were flushed from mouse tibia and femur and cultured in AIM-V containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 1% antibiotic/antimycotic (Invitrogen, Carlsbad, CA), and were cultured in AIM-V containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 1% antibiotic/antimycotic (Invitrogen) and supplemented with 50 ng/mL mGM-CSF (R&D Systems, Minneapolis, MN) and 10 ng/mL mIL-4 (R&D Systems) for 3 d. Cells were cultured in a humidified chamber at 37°C and 5% atmospheric CO$_2$. The culture medium was removed and replenished with an equal volume of fresh medium and cytokines on days three and five. The immature DC were harvested on day six and pulsed with mRNA and lysate derived from the original luc2-negative Kras$^{G12D}$/p53$^{-/-}$/C14 parental cell line as described previously. Briefly, lysate was prepared by resuspension of tumor cells at 2 $\times 10^7$/mL in PBS followed by three rapid freeze/thaw cycles in liquid nitrogen and a 37°C water bath. Freeze/thawed lysate was stored at $-20^\circ$C until use. Antigen-pulsed DC were matured using a cocktail of 50 ng/mL GM-CSF, 10 ng/mL IL-4, 10 ng/mL IL-1f (R&D Systems), 10 ng/mL TNF-$\alpha$ (R&D Systems), 15 ng/mL IL-6 (R&D Systems), and 1 $\mu$g/mL PGE$_2$ (Sigma-Aldrich, St. Louis, MO). Unused mature DC were cryopreserved for future use.

**Vaccination and chemotherapy**

Three days post tumor implantation, mice received primary vaccination with 200,000 DC per mouse i.p. The TLR-7 agonist Imiquimod was given as an adjuvant during primary vaccination. A booster injection of DC was given 10 d later. The mice in the combination therapy and drug monotherapy groups received 40 mg/kg gemcitabine i.p. for two weeks every alternate day starting on day 13 post-implantation.
Post tumor implantation, mice were injected with 100 µL of 10 mg/mL D-Luciferin (Regis Technologies, Morton Grove, IL), incubated for 10 min and bioluminescence was measured with the IVIS imaging system (Caliper Life Sciences, Waltham, MA) after a 30 sec exposure. Imaging was done at weekly intervals. On post-inoculation day 181, surviving mice that received combination therapy (4/4) or drug monotherapy (3/5) were retro-orbitally re-challenged with 500,000 KrasG12Dp53−/− cells. Tumor growth was monitored by IVIS imaging. Five naive mice were included as controls.

**Analysis of circulating lymphocytes**

Mice were bled retro-orbitally on days 49 and 101 post tumor implantation. Red blood cells were lysed by treatment with ammonium chloride (Sigma-Aldrich) as recommended by the manufacturer’s instructions. The white blood cell pellet was washed once with PBS and resuspended in AIM-V medium + 10% mouse serum. Cryopreserved, loaded DC were thawed and washed and co-cultured overnight with the fresh PBMC in a 96 well plate at a ratio of 1:10. The following day, cells were stained with anti-CD3, CD8, CD25, IFNγ, NK1.1, CCR7, Lag3, and GITR for analysis by flow cytometry. All flow cytometric analysis was performed using an LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo version 10.0.00003 (Tree Star Inc., Ashland, OR) for OS-X.

**Histology and immunohistochemistry**

Intact tissues were fixed in 10% formalin for 24 h, rinsed with 70% ethanol, embedded in paraffin, and sectioned in 5 µm increments. Paraffin sections were stained with hematoxylin and eosin (H&E) for gross histological analysis by light microscopy using an Olympus CX41 microscope (Olympus Corporation, Center Valley, PA) with an Olympus DP70 digital camera (Olympus Corporation).

**Splenocyte adoptive transfer**

On post-inoculation day 259, splenocytes were harvested from the mice that received the combination therapy and survived both the primary challenge and the secondary retro-orbital rechallenge. Non-adherent lymphocytes were separated from the monocytic fraction by plastic adherence, and CD8⁺ cells were negatively selected by magnetic separation (Miltenyi-Biotec, San Diego, CA). Purified CD8⁺ cells were activated by overnight culture with PDAC antigen loaded DC at the ratio of 1 DC per 10 T-cells. The following day, NK1.1₅⁴ and NK1.1₅⁸ populations were separated on a FACSAria Ilu, (BD Biosciences, San Jose, CA), and 1,500 sorted cells were adoptively transferred by i.p. administration into naive mice. 250,000 KrasG12Dp53−/− tumor cells were injected intraperitoneally the following day.

**Western blotting and analysis**

All gel electrophoreses was performed under denaturing, reducing conditions on a 12% polyacrylamide gel with subsequent transfer to a 0.45 µm nitrocellulose membrane for antibody probing. All blocking and antibody staining steps were carried out in 5% milk, and primary antibodies were applied overnight at 4°C. Western blot chemiluminescent signal was detected using a ChemiDoc XRS digital imaging system supported by Image Lab software Version 2.0.1 (Bio-Rad Laboratories, Hercules, CA). All Western blots were quantitated by densitometry of Ponceau S (Sigma-Aldrich) stained membranes. Contamination of supernatants with residual cell lysate or debris from cell death was controlled for by immunostaining with anti-β-actin (Santa Cruz) and additional densitometry. Densitometry was performed using ImageJ software (NIH, Bethesda, MD). All western blots are representative of at least three independent experiments.

**RT-PCR**

Total RNA was extracted from the DC by means of the Trizol method and cDNA synthesis was performed using RT PCR using High-Capacity cDNA Reverse Transcription Kit (Life Technologies) according to the manufacturer’s instructions. Real time PCR was performed with the 7500 Real-time PCR system (Applied Biosystems, Foster City, CA) using the Taqman Realtime PCR assay (ThermoFisher Scientific, Waltham MA) according to the manufacturer’s instructions. The primers used were IL-1β (Mm00434228_m1, FAM), IL-6 (Mm00446190_m1, FAM), IL-12a (Mm00434165_m1, FAM), Ctl4 (Mm01253995_m1, FAM), and 18s rRNA (4319413E, VIC).

**Statistical analysis**

Statistical significance was determined by the two-tailed Student’s t test or ANOVA using Prism Software (GraphPad Software, La Jolla, CA). Survival was analyzed according to the Kaplan–Meier method. Bonferoni correction was applied when necessary to control for type I errors during multiple comparisons. Data are presented as the mean ± SEM unless stated otherwise. Statistical significance was defined as p ≤ 0.05.

**Results**

**Dendritic cells loaded in a homologous fashion with PDAC antigens exhibit hallmarks of the TH1 DC phenotype**

As a first step in the development of a T₄₁ DC vaccine, DC were loaded with mRNA and lysate derived from KrasG12Dp53−/− luc2neg cells, matured, and analyzed for previously described hallmarks 16–18 of homologous antigenic loading. Also as described previously, 16–18 DC were loaded singly with PDAC mRNA, singly with PDAC cell lysate, and doubly in a heterologous fashion with PDAC mRNA and irrelevant B16 tumor lysate to serve as comparative controls. Western blot of the DC lysates probed with AIMp1 antibody indicated that only homologous antigenic loading resulted in significantly elevated levels of AIMp1 production (Fig. 1A).
Gemcitabine treatment in combination with T\textsubscript{h}1 DC vaccination mediates tumor eradication

Using an aggressive therapeutic model system, \(1.0 \times 10^6\) \(\text{Kras}^{G12D}\text{p53}\text{-}^{-}\) PDAC tumor cells were orthotopically implanted into the pancreata of wild type C57BL/6 mice after which mice were treated therapeutically with gemcitabine (40 mg/kg) alone, T\textsubscript{h}1 DC vaccination alone, or gemcitabine in combination with a T\textsubscript{h}1 DC vaccine. Though median survival was identical for untreated controls, mice treated with gemcitabine alone, and mice treated with vaccination alone, 100% of mice treated with a combination of gemcitabine and vaccination remained alive through study day 53 (Fig. 1D, \(p = 0.003\)).

Given these unexpected results, a second therapeutic model system was constructed to comprehensively characterize this phenomenon. In this model system, \(0.5 \times 10^6\) \(\text{Kras}^{G12D}\text{p53}\text{-}^{-}/\text{luc}2\) PDAC tumor cells were orthotopically implanted into the pancreata of wild type C57BL/6 mice after which mice were treated therapeutically with gemcitabine (40 mg/kg) alone or gemcitabine in combination with T\textsubscript{h}1 DC vaccination (treatment schema outlined in Fig. 2A). Tumor growth was monitored weekly by IVIS imaging, and by day 32 post-implantation, all treated mice were in remission. However, by day 85, 80% of mice treated with gemcitabine alone had relapsed whereas all animals treated with gemcitabine + vaccine remained in complete remission. All the mice that received the combination therapy survived in complete remission through study day 180 whereas 40% of mice treated with gemcitabine alone had died and 40% were alive with progressive disease (Fig. 2B and C).

Combination therapy results in higher levels of activated, antigen-specific CD8\textsuperscript{+} T-cells

To determine if the durable remissions imparted by combination therapy could be correlated with identifiable immunologic parameters, peripheral blood was collected retro-orbitally from mice on post-inoculation days 49 and 109, and T-cells were analyzed by flow cytometry after overnight culture with either
PDAC antigen-loaded or PDAC antigen-naive DC stimulators. When day 49 T-cells were cultured with PDAC antigen-loaded DC, those derived from mice treated with combination therapy exhibited exceptionally elevated levels of activated IFN-γ producing CD8+ T-cells as well as elevated levels of atypical CD3+CD8+NK1.1+ T-cells (Fig. 3A). These same phenomena were observed among day 109 T-cells as well as the upregulation of CD8+CCR7+ T-cells and marked downregulation of regulatory CD8+GITR+ T-cells in the mice that received combination therapy (p < 0.05. **p < 0.01. Error bars ± SD).

**Combination therapy provides ongoing and durable protection against tumor**

To determine the durability of the antitumor immune response imparted by various treatment regimens, mice that remained alive on day 181 post-inoculation were re-challenged retro-orbitally with 500,000 KrasG12D;P53−/−Luc2+ cells. A group of five naive mice were also retro-orbitally challenged as a control. All mice in the drug monotherapy group that had survived their original PDAC inoculation (including a mouse that was NED) as well as all naive mice rapidly developed significant cranial tumor burden and died whereas only 25% of mice that had received combination therapy exhibited any evidence of cranial tumor burden (Figs. 4A and 5A–C) and none died (Fig. 4B). Postmortem histopathology showed extensive lung metastases in all naive mice and mice treated with gemcitabine monotherapy as well as sporadic metastases to other organs including spleen and heart. In contrast, no metastases were observed among any mice that had received combination therapy (Fig. 5D–F). Overall, the results indicated that TH1 DC vaccine in combination with gemcitabine could not only eradicate tumor but also provide durable protection against relapse and metastasis.

**CD8+ NK1.1+ splenocytes mediate robust and durable immunological memory**

Analysis of peripheral blood lymphocytes revealed a significant correlation between levels of CD3+CD8+ NK1.1+ cells and survival (i.e., Fig. 3). To determine if durable immunologic memory could be transferred along with this cell type, non-adherent CD8+ splenocytes were negatively selected from the spleens of mice treated with combination therapy 260 days prior. These cells were activated overnight with PDAC-loaded DC...
stimulators after which responding NK1.1pos and NK1.1neg cells were separated by flow sorting. Scant populations of 1,500 NK1.1pos and NK1.1neg cells were adoptively transferred into each of three naive recipient mice in conjunction with i.p. PDAC challenge using 250,000 KrasG12Dp53−/−/luc2 cells. Tumor growth was subsequently measured and quantified by IVIS imaging. The data (Fig. 6A and B) indicated that anti-tumor immunity could be transferred from vaccinated host to naive recipient by very small numbers of CD8+ NK1.1+ cells, indicating that this cell type can mediate durable antitumor protection and memory responses in this model of pancreatic cancer. Mice that received CD8+ NK1.1+ cells remained in permanent remission (Fig. 6C) with NED (no evidence of disease).

Discussion

Despite decades of research, PDAC remains the malignancy with the most abysmal of prognoses. Vaccine immunotherapy has long been anticipated to eradicate cancer in a targeted, non-toxic fashion but meaningful success up to now has been elusive in clinical studies. DC-based vaccination has previously been shown to be effective in some murine models of pancreatic cancer. Several studies have reported that DC vaccination of tumor bearing mice has led to increased tumor specific lysis by CD8+ T cells, expansion of IFNγ secreting T cells, and tumor regression. Targeting primary or metastatic lesions with intra-tumoral DC was effective in murine pancreatic cancer models and could be beneficial in human trials since a majority of patients are unresectable at the time of diagnosis. In one open label clinical trial, intra-tumoral injection of DC led to enhanced immunity and regression of tumor in several patients with pancreatic cancer. Although clinical trials have demonstrated that tumor-specific immunity can be regularly established by DC-based vaccines, it has also become evident that clinical responses to immunotherapy occur only rarely in patients with gastrointestinal malignancies, and the field in recent years has tried to identify strategies that, not only overcome tumor-induced immunosuppression, but also do not interfere with the activation of a tumor-directed immune response. Increasing evidence suggests that well established treatment strategies such as radiation, surgical debulking, or chemotherpay can be successfully combined with immunotherapeutic approaches. Here, we show that gemcitabine chemotherapy augments the therapeutic efficacy of T11 DC vaccination in a murine model of PDAC. Gemcitabine has paradoxically been reported to enhance immunocompetence in certain PDAC patient populations via various mechanisms...
including selective deletion of myeloid-derived suppressor cells (MDSC) that can inhibit antitumor immunity.\textsuperscript{31-33} A previous study also found that gemcitabine can induce the proliferation of CD14\textsuperscript{+} monocytes and CD11c\textsuperscript{+} DC, findings that could provide a rationale for the combination of gemcitabine and specific immunotherapy.\textsuperscript{34} When used in combination with the standard of care chemotherapy drug gemcitabine, DC vaccination was previously shown to increase tumor free survival in a murine model of pancreatic cancer.\textsuperscript{35} In the present study, the combination of T\textsubscript{H1} DC vaccination and gemcitabine chemotherapy resulted in significant improvement in tumor-free survival in the p53\textsuperscript{-/-}/Kras\textsuperscript{G12D}\textsuperscript{+}\textsuperscript{-} murine tumor model of PDAC. This is in accordance with previous findings in which gemcitabine was shown to augment the effectiveness of immune stimulation by in vivo CD40 ligation.\textsuperscript{36} In a human in vitro model, gemcitabine was shown to sensitize pancreatic carcinoma cells to CTL responses.\textsuperscript{37} Apoptosis induced by gemcitabine was also shown to increase cross-presentation of tumor antigens to CTLs by intra-tumoral DCs.\textsuperscript{38} Although inhibition of B cell-mediated immune responses has been described in animal models, gemcitabine can be administered to patients with pancreatic cancer without relevant loss of T cell and DC function.\textsuperscript{39} Moreover, in cancer patients, gemcitabine may even inhibit T\textsubscript{H2}- and specifically augment T\textsubscript{H1}-type immune responses.\textsuperscript{39} Studies by Beatty et al.\textsuperscript{40} have also shown therapeutic effects of gemcitabine treatment with CD40 agonist, CP-870,893 in patients with metastatic PDAC.

We have recently characterized novel and critical biological parameters that govern cell-mediated immune responses and have applied these concepts to PDAC immunotherapy. Our substantial preliminary dataset indicates that orthotopically injected Kras\textsuperscript{G12D}\textsuperscript{+}\textsuperscript{-} PDAC tumors can be completely eradicated through a combination of T\textsubscript{H1} DC vaccine and conventional gemcitabine whereas gemcitabine alone could not prevent tumor recurrence. These results were due in part to enhanced induction of non-classical PDAC-specific CD8\textsuperscript{+}NK1.1\textsuperscript{+} T-cells among vaccinated animals. NKR-P1 is a family of disulfide-linked homodimers of which NK1.1 (CD161 in humans) is a member.\textsuperscript{40} NKR-P1A and C are activating molecules that can trigger cytokine production and cytolytic activity by NK cells,\textsuperscript{41,42} while NKRP1B appears to be inhibitory.\textsuperscript{43,44} The PK136 antibody recognizes the NKR-P1C receptor (NK1.1) in the mouse. This antibody depletes NK cells in vivo\textsuperscript{45} and induces activation and proliferation of both NK and NKT cells in vitro.\textsuperscript{46,47} Assarsson et al.\textsuperscript{48} first described the presence of NK1.1\textsuperscript{+}TCR\beta\textsuperscript{+} cells within lymphokine-activated killer cell cultures derived from CD1d\textsuperscript{1+/-} and Ja281\textsuperscript{1+/-} C57BL/6 mice that lack classical NKT cells. Unlike classical NKT cells, 50-60\% of these NK1.1\textsuperscript{+}TCR\beta\textsuperscript{+} cells expressed CD8\textsuperscript{+} with a diverse TCR repertoire. Upon in vitro stimulation with IL-2, IL-4, or IL-15, purified NK1.1\textsuperscript{+} CD8\textsuperscript{+} T-cells rapidly acquired surface expression of NK1.1. The induction of NK1.1 on CD8\textsuperscript{+} T cells was not just an in vitro phenomenon as Assarsson also observed a five-fold increase of NK1.1\textsuperscript{+}CD8\textsuperscript{+} T cells in the lungs of influenza virus-infected mice. These data suggest that CD8\textsuperscript{+} T cells can acquire NK1.1 and other NK cell-associated molecules upon appropriate stimulation in vitro and in vivo. T-cell responses to influenza were reported to be
deficient in mice treated with the anti-NK1.1 monoclonal antibody. In the intervening years, the analogous population of CD3+CD8+CD161+ T-cells has been described in human populations, though never in the context of cancer or tumor immunity. These cells are described in the infectious disease literature as highly cytotoxic memory T-cells, often with antiviral specificity. Given that T411 DC vaccination mimics an antigenic micro-environment present only in the context of viral infection, the appearance of such T-cell responses might be anticipated.

In summary, we demonstrate that adjuvant T111 DC vaccination can lead to durable cure of KrasG12Dp53−/− PDAC when applied in conjunction with standard of care gemcitabine chemotherapy. Durable tumor immunity was correlated with the appearance of atypical CD8+NK1.1+ cells, a memory cell previously described in the context of viral infection but not in cancer. Additional work will be necessary to delineate the role of T411 vaccination in the development of these novel anticancer responses.

Disclosure of potential conflicts of interest
WKD and MMH hold ownership stakes in Diakonos Research, Ltd. All other authors declare no competing financial interests.

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Author contributions
VK, QCY, and WKD conceived of and designed the study. VK, DLi, MMH, DLiang, ZL, and YC acquired data. VK, DLi, MMH, WEF, JML, 490 SP, QCY, and WKD analyzed and interpreted data. VK and WKD drafted the manuscript. MMH, WEF, JML, and QCY critically revised the manuscript for important intellectual content. VK and WKD provided statistical analysis. QCY and WKD obtained funding, provided material support, and supervised the study. JML provided technical support.

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