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Stromal Modulation Reverses Primary Resistance to Immune Checkpoint Blockade in Pancreatic Cancer

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) remains one of the most difficult cancers to treat. It is refractory to most existing therapies, including immunotherapies, due to the presence of an excessive desmoplastic stroma, which restricts penetration of drugs and cytotoxic CD8⁺ T cells. Stromal modulation has shown promising results in the enhancement of immune checkpoint blockade treatment in PDAC. We here demonstrate effective stromal modulation by a polymeric micelle-based nanoformulation to co-deliver a sonic hedgehog inhibitor (cyclopamine, abbreviated as CPA), and a cytotoxic chemotherapy drug (paclitaxel, abbreviated as PTX). The formulation, M-CPA/PTX, modulated the PDAC stroma by increasing the intratumoral vasculature density, which then promoted the tumor infiltration by cytotoxic CD8⁺ T cells without depletion of tumor-

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SUPPORTING INFORMATION

Supporting Information is available free of charge *via* the Internet at <http://pubs.acs.org>: Representative images of tumor-bearing mice and resected tumors. Gating strategies for flow cytometry analyses of immune cells. Additional RT-PCR, immunoblotting, and cytokine analysis of treated tumors or cancer cells. Representative immunofluorescence staining and flow cytometry plots. Heat map of RPPA analyses.

restraining α -smooth muscle action-positive fibroblasts and type I collagen in the stroma. The combination of M-CPA/PTX and PD-1 checkpoint blockade significantly prolonged animal survival in an orthotopic murine PDAC model as well as a genetically engineered mouse model (GEMM) of PDAC. The superior anti-tumor efficacy was mediated by enhanced tumor infiltration of CD8⁺ T cells without concomitant infiltration of suppressive regulatory T cells (Tregs) or myeloid-derived suppressor cells (MDSCs), and by the coordinated action of PTX and interferon-gamma (IFN- γ). Our results demonstrate that stroma-modulating nano-formulations are a promising approach to potentiate immune checkpoint blockade therapy of pancreatic cancer.

Graphical Abstract

Keywords

pancreatic cancer; immune checkpoint blockade; tumor-associated stroma; immune suppression; polymeric micelles

PDAC remains one of the most difficult cancers to cure. The 5-year overall survival rate of patients with pancreatic ductal adenocarcinoma (PDAC) has remained around 5% during the last several decades in spite of vigorous research.¹ Therefore, there is an urgent unmet need to identify more effective therapies against PDAC. Recently, immune checkpoint blockade (ICB) has shown promise in the treatment of cancer patients, producing outstanding responses in a variety of cancers.² The efficacy of ICB in patients with PDAC, however, has been limited.³ We here explore a combination therapy to improve the efficacy of ICB against PDAC.

ICB activates CD8⁺ cytotoxic T lymphocytes (CTLs) by blocking inhibitory checkpoint molecules with antibodies including anti-CTLA-4, anti-PD1, and anti-PD-L1, resulting in CTL-mediated tumor control.^{4, 5} Clinical data suggest that patients with pre-existing tumor infiltrating CTLs are most likely to benefit from ICB therapy.^{6, 7} However, the PDAC stroma, which is highly fibrotic and contains few blood vessels, imposes a formidable physical barrier to CTL infiltration and results in a highly immunosuppressive tumor microenvironment.^{8, 9}

Recent studies on modulation of the PDAC stroma to enhance immunotherapy have generated mixed results. The efficacy of anti-PD-L1 was enhanced by genetically depleting fibroblast activation protein- α -positive (FAP- α ⁺) cancer-associated fibroblasts.¹⁰ The antitumor activity of anti-CTLA-4 was enhanced by depleting α -smooth muscle actin-positive (α -SMA⁺) cancer-associated fibroblasts.¹¹ However, extensive depletion of cancer-

associated fibroblasts in the PDAC stroma also led to infiltration by immunosuppressive regulatory T cells, depletion of type I collagen, and induction of highly aggressive tumor phenotypes.^{11, 12} Treatment of the PDAC stroma with PEGylated hyaluronidase-degraded hyaluronic acid improved the intratumoral delivery of chemotherapy drugs¹³ and may allow more effective tumor infiltration by CTLs.¹⁴ This approach, however, is limited to patients with high tumor levels of hyaluronic acid.¹⁵ Taken together, these studies demonstrate that it is feasible to enhance the efficacy of ICB in PDAC by stromal modulation. The challenge, however, is to identify a strategy that can simultaneously enhance tumor infiltration by activated CTLs and maintain the tumor-restraining collagenous matrix of PDAC. To our knowledge, there is no report on using pharmacologic, instead of genetic, approaches to achieve these goals.

Sonic hedgehog (SHH) is upregulated in the majority of PDAC cases, and SHH activates the cancer-associated fibroblasts in a paracrine manner to produce a highly fibrotic stroma.^{16, 17} Both genetic and pharmacologic approaches have been adopted to disrupt SHH signaling in PDAC. Although SHH inhibition increased intratumoral vasculature and subsequently improved drug delivery, the collateral ablation of the collagenous matrix promoted unchecked proliferation of tumor cells.¹⁸ Pathological analyses revealed an undifferentiated phenotype of tumor cells that grew aggressively with SHH inhibitors, leading to disappointing results in both preclinical and clinical studies.^{19, 20} As an alternative, we developed a nanoformulation (M-CPA/PTX, Figure 1) using biodegradable polymeric micelles to encapsulate cyclopamine (CPA), a SHH inhibitor, and paclitaxel (PTX), a potent chemotherapy drug.²¹ When PDAC was treated with M-CPA/PTX, tumor cell proliferation was restrained by PTX while SHH signaling was disrupted by CPA.²¹ Most importantly, a functional vasculature was restored without the formation of an aggressive undifferentiated tumor phenotype.²¹

Given the known beneficial effects of vascular normalization on T cell infiltration into tumors, we hypothesized that M-CPA/PTX could promote CTL infiltration and thereby enhance ICB in PDAC. The preclinical results reported herein demonstrate that M-CPA/PTX increased tumor infiltration by CTLs and overcame resistance to anti-PD1 therapy in an orthotopic murine PDAC model and a genetically engineered mouse model (GEMM) of PDAC (the KPC-Luc model). Further analyses revealed that sensitization of PDAC to anti-PD1 could be attributed to a coordinated action on tumor cells by intratumoral PTX and interferon-gamma (IFN- γ). Our data demonstrate that co-delivery of PTX and a stroma-modulating agent in a nanoformulation is a promising approach to sensitizing PDAC to ICB therapy.

Results and Discussion

Polymeric micelles co-loaded with CPA and PTX

The detailed preparation and characterization of the polymeric micelles loaded with CPA and PTX (M-CPA/PTX) have been reported in our previous publication.²¹ In brief, the micelles were self-assembled from an anionic and a cationic block copolymer in the presence of CPA and PTX (Figure 1). The anionic polymer consisted of a brush-like poly(ethylene glycol) block and a brush-like poly(ϵ -caprolactone) block. The end of each

oligo(ϵ -caprolactone) side-chain was extended with succinic anhydride to produce a residual carboxylic acid, which then interacted with the secondary amine of CPA to stabilize the micellar core. The cationic polymer consisted of a brush-like block with poly(ethylene glycol) and a brush-like block with pendent quaternary ammonium cations. PTX was simultaneously encapsulated along with CPA. The resultant M-CPA/PTX was spherical and had a diameter in the range of 50 to 100 nm (Figure 1). M-CPA/PTX showed excellent stability. During 90-day storage, there was no significant change in micelle diameter or levels of drug loading, and there was only a slight increase in the polydispersity index (Table 1).

M-CPA/PTX modulated tumor-associated stroma in favor of immunotherapy

Previously, we have demonstrated that CPA encapsulated in M-CPA/PTX contributed to the inhibition of Hedgehog signaling and stemness pathway in the KPC-Luc PDAC model.²¹ In the current study, we first examined how M-CPA/PTX modulated stroma in the Kras* orthotopic murine PDAC model. Kras* cells exhibit a doxycycline-driven mutation of Kras^{G12D} oncogene, which is a dominant driver of PDAC initiation and progression.²² Tumor microvessel density (MVD, expressed as percentage of CD31⁺ pixels per visual field) was measured at different times after initiation of M-CPA/PTX treatments (Figure 2A). MVD on day 2 was similar to that of control ($0.9 \pm 0.1\%$ vs. 1.0 ± 0.2 , $p > 0.05$), which then increased significantly to $2.0 \pm 0.2\%$ on both day 4 and day 6 ($p < 0.0001$ vs. control) (Figure 2B). Immunoblotting revealed significant increase in the phosphorylation of vascular endothelial growth factor receptor 2 (p-VEGFR2_Y1175) and Src family proteins (p-Src_Y416); as well as a significant decrease in the phosphorylation of p38 MAP kinase (p-p38 MAPK_T180/Y182, Figure 2C). There was no significant changes among the other components of the VEGFR2-dependent angiogenesis signaling. Phosphorylation of VEGFR2 is a hallmark for VEGF-related angiogenesis,²³ while the activation of Src proteins can improve vessel permeability.²⁴ On the other hand, inhibition of p38 MAPK can increase the VEGF-induced sprouting angiogenesis.²⁵ Therefore, our data suggest that the M-CPA/PTX-induced increase in MVD is attributed to VEGF-mediated angiogenesis.

The protein expression of Shh ligand, a component of Hedgehog signaling, was significantly reduced by 25%, 53%, and 61% on days 2, 4, and 6, respectively ($p < 0.01$ vs. control, Figure 2D), proving that Hedgehog signaling was disrupted in the Kras* tumor. Real-time polymerase chain reaction (RT-PCR) also confirmed the decrease of Hedgehog signaling (*Gli-1*, *Shh*, and *Ptch1*), stemness (*Nanog* and *Aldh1a1*), and hypoxia at the mRNA level (*Caix*, Figure S1). The protein expression of carbonic anhydrase 9 (CA-IX), a hypoxia marker, decreased to $56.2 \pm 0.2\%$ that of control on day 6 ($p < 0.001$). In addition, FAP- α expression was reduced to 60% that of control starting from day 2 ($p < 0.0001$). Normalization of intratumoral vasculature is known to promote tumor infiltration by immune cells.²⁶ Both hypoxia and the presence of FAP- α -expressing cancer-associated fibroblasts are important contributors to immunosuppression.^{10, 27} Our data indicate that M-CPA/PTX modulated the stroma of Kras* tumor in favor of immunotherapy.

M-CPA/PTX potentiated anti-PD1 therapy in Kras^{*} orthotopic murine PDAC model

We next investigated whether the stromal modulation by M-CPA/PTX could potentiate anti-PD1 therapy. Treatments were initiated once tumor became palpable (~5 mm in diameter) using the schedule outlined in Figure 3A. Anti-PD1 was intraperitoneally injected starting from 2 days after the completion of M-CPA/PTX; because, as shown in Figure 2B, the intratumoral vasculature began to significantly increase from this time point. M-CPA/PTX or anti-PD1 monotherapies did not significantly improve animal survival compared to the untreated control. The median survival after enrollment was 16.5 days for control and M-CPA/PTX and 23 days for anti-PD1. In contrast, the median survival of mice treated with the combination of M-CPA/PTX and anti-PD1 was 31 days, significantly longer than the survival of any of the other 3 groups (log-rank test, $p < 0.0001$) (Figure 3B).

In order to interrogate the treatment-induced changes in the tumor and stromal compartments, we treated mice using the schedule outlined in Figure 4A and collected tumor tissues after the last dose of anti-PD1 (day 10). There was no significant decrease in body weight in any of the treated groups (Figure 4B). Tissue section of normal organs showed no signs of toxic reactions either (Figure S2). Representative images of resected tumors are shown in Figure S3. The tumor weight of the combination therapy group (0.39 ± 0.04 g) was about half that of the other 3 treatment groups ($p < 0.05$, Figure 4C), consistent with the Ki67 staining of proliferating cells (Figure 4D), and CD31 staining of the MVD (Figure 4E). These data suggest that anti-PD1 contributed to suppression of tumor cell proliferation and increase of angiogenic activity. In contrast, there was no significant difference between treatment groups in the fibrotic components of Kras^{*} tumor stroma, including type I collagen (Picrosirius red staining, Figure 4F) and α -SMA⁺ cells (Figure 4G). These data are consistent with our previous report,²¹ that tumor stromal modulated by M-CPA/PTX, alone or in combination with anti-PD-1 ICB, did not compromise the critical tumor-restraining function of the extracellular matrix.

Combination of M-CPA/PTX and anti-PD1 increased intratumoral levels of CD8⁺ T cells and IFN- γ

To investigate the possible mechanism(s) of enhanced antitumor activity with M-CPA/PTX and anti-PD1, we next profiled the intratumoral immune cells in Kras^{*} tumors from mice treated with each monotherapy and the combination therapy as outlined in Figure 4A. Representative flow charts and gating strategies are summarized in Figures S4 and S5. The number of CD8⁺ T cells per gram of tumor was significantly higher in the M-CPA/PTX + anti-PD1 group than in the other 3 groups (Figure 5A). Correspondingly, we observed increased mRNA expression levels of IFN- γ and PD-L1 in Kras^{*} tumors treated with anti-PD1 and M-CPA/PTX + anti-PD1 than in Kras^{*} tumors treated with M-CPA/PTX and tumors from untreated controls (Figure S6), consistent with previous work linking IFN- γ and PD-L1 expression with the presence of CD8⁺ T cells.²⁸ We also observed increased mRNA expression level of CTLA-4 after combination therapy (Figure S6), suggesting that addition of anti-CTLA-4 to the M-CPA/PTX + anti-PD1 regimen may further enhance antitumor efficacy against PDAC because anti-PD1 and anti-CTLA-4 target 2 different, non-redundant immune checkpoint pathways.²⁹ We did not detect significant differences between the monotherapies and control or between the combination therapy and control in the

numbers of regulatory T cells (Treg), myeloid-derived suppressive cells (MDSCs), macrophages, or dendritic cells (Figure 5). However, M-CPA/PTX alone lowered the number of these immune cells compared to the combination therapy ($p < 0.05$) (Figure 5), possibly reflecting the cytotoxic effect of taxanes on these immune cells as observed after M-CPA/PTX monotherapy. Therefore, compared to untreated control, the combination therapy increased tumor infiltration by CD8⁺ T cells (3 times as high in the combination therapy group as in the control group, $p < 0.01$, Figure 5A) without significant recruitment of regulatory T cells (Figure 5B) or MDSCs (Figure 5C). These results suggested that the selectively increased tumor infiltration by CD8⁺ T cells, which was not observed with either M-CPA/PTX or anti-PD1 alone, may be responsible for the enhanced antitumor activity of M-CPA/PTX + anti-PD1 against PDAC.

Previously, Zhong *et al.*³⁰ showed that pretreatment with low-dose PTX (2 mg/kg PTX, single injection) modulated the intratumoral cytokine network, as indicated by increased intratumoral levels of CCL2 (MCP-1) and CXCL10 (IP10) 11 days after initiation of treatment. To investigate whether the M-CPA/PTX regimen used in our study (3 doses of M-CPA/PTX at 5 mg/kg PTX per dose) also modulated the intratumoral cytokine network, we used Luminex technology to analyze an array of 15 cytokines in tumors collected on day 10 after initiation of treatment. We found that M-CPA/PTX alone did not change the intratumoral level of any of the cytokines analyzed in Kras* PDAC tumors, including CCL2 and CXCL10 (Figure 5 and Figure S7). The combination therapy also did not have significant effects on most of the cytokines analyzed with the exception of IFN- γ and CCL5 (Figure 5 and Figure S7). Intratumoral levels of IFN- γ and CCL5 in the combined M-CPA/PTX + anti-PD-1 were significantly higher than that in the un-treated control and the M-CPA/PTX alone groups. However, the combination therapy only moderately increased the level of IFN- γ compared to anti-PD alone ($p = 0.11$). Similarly, we did not observe differences in CCL5 between anti-PD1 and combined M-CPA/PTX + anti-PD1. CCL5, also known as RANTES (regulated on activation, normal T cell expressed and secreted), plays an active role in recruiting CD8⁺ T cells to melanoma.^{31, 32} Increased production of the chemokine CCL5 has not previously been reported in PDAC tumors after treatments with checkpoint inhibitors. Together, these data imply that the tumor wide increases in levels of IFN- γ and CCL5 in Kras* PDAC treated with the combination of M-CPA/PTX and anti-PD1 were mainly a contribution of anti-PD1 treatment.

CD8⁺ T cells and IFN γ are indispensable for antitumor efficacy of M-CPT/PTX + anti-PD1

Because we observed increased CD8⁺ T cells and IFN- γ in tumors treated with M-CPA/PTX + anti-PD1, we hypothesized that CD8⁺ T cells and IFN- γ were responsible for the enhanced antitumor activity of combined M-CPA/PTX + anti-PD1. Indeed, depletion of CD8⁺ T cells by anti-CD8 antibody reduced the median survival of tumor-bearing mice treated with M-CPA/PTX + anti-PD1 from 31 days to 14.5 days ($p < 0.0001$) (Figure 6A). Moreover, tumor-suppressive effect of combined M-CPA/PTX + anti-PD1 was abolished after administration of anti-IFN- γ antibody to deplete IFN- γ (Figure 6B). At 6 days after the start of treatment, the tumors in the IFN- γ depletion group were significantly larger than those in the combination therapy group ($252 \pm 36 \text{ mm}^3$ vs. $131 \pm 25 \text{ mm}^3$, $p < 0.05$). The difference was even greater at 23 days after the start of treatment (1780 ± 125 vs. 466 ± 172

mm³, $p < 0.0001$). A similar trend in tumor size was also observed in orthotopic Kras* model after neutralization of IFN- γ (Figure S8). Thus, IFN- γ and CD8⁺ T cells were indispensable for the antitumor efficacy of combined M-CPA/PTX + anti-PD1.

Given that T-cell exclusion from the vicinity of tumor cells is one of the major factors limiting the therapeutic efficacy of ICB,²⁶ we investigated the tumor penetration of CD8⁺ T cells and the intratumoral spatial distribution of IFN- γ using immunohistochemical (IHC) staining (Figures. 6C & 6D). The M-CPA/PTX + anti-PD1 group had a significantly higher frequency of CD8⁺ T cells in the vicinity of tumor cells (84 ± 7 cells per visual field) than anti-PD1 (30 ± 4 , $p < 0.0001$), M-CPA/PTX (53 ± 7 , $p < 0.01$), and untreated control (20 ± 4 , $p < 0.0001$) (Figure 6C). The M-CPA/PTX group had significantly more tumor-penetrating CD8⁺ T cells than the untreated control. Moreover, immunofluorescence staining of CD8 α and CD31 revealed a prominent extravasation of CD8⁺ T cells from blood vessels (Figure S9). The intratumoral concentration of IFN- γ (expressed as mean fluorescence intensity, Figure 6D) was significantly higher in the combination therapy group (13.4 ± 1.9 a.u.) than in the anti-PD1 (5.7 ± 1.0 a.u., $p < 0.001$), M-CPA/PTX (1.5 ± 0.3 a.u., $p < 0.0001$), or untreated control group (1.1 ± 0.6 a.u., $p < 0.0001$). These data indicate that M-CPA/PTX modulated tumor stroma in such a way that it overcame T-cell exclusion and permitted penetration of CD8⁺ T cells to the vicinity of tumor cells.

PTX and IFN- γ acted coordinately to inhibit pancreatic cancer cell growth and induce apoptosis at low PTX concentrations

Analysis of intratumoral drug concentration showed that at 24 hours after the last dose of M-CPA/PX, CPA concentration was at 1.2 ± 0.2 μ M, and PTX concentration was at 2.3 ± 0.3 μ M (Figure S10). Therefore, both CPA and PTX were successfully delivered to Kras* tumor. Moreover, the elevated level of intratumoral IFN- γ in the vicinity of tumor cells in the combined M-CPA/PTX + anti-PD1 group may exert direct cell killing activity to potentiate the cytotoxic effect of PTX that had been successfully delivered to the tumor by M-CPA/PTX.²¹ To address this possibility, we investigated if IFN- γ and PTX worked in concert to kill cultured Kras* PDAC cells. We did not include CPA in this study because CPA or micelle-encapsulated CPA did not show significant cytotoxicity against Kras* cells (half-maximal inhibitory concentrations [IC₅₀] > 10 μ M, Figure S11A). IFN- γ alone only modestly lowered cell viability, which was reduced by 28% to 44% over a IFN- γ concentration of 0.3 to 1000 ng/mL (Figure 7A). In contrast, Kras* cells were sensitive to PTX; the IC₅₀ of PTX in inhibiting Kras* cell viability was 4.7 ± 0.7 nM. (Figure 7B). For the combination treatment with PTX and IFN- γ (Figure 7C), adding IFN- γ significantly reduced cell viability at PTX concentrations below 10 nM, but had no further impact on cell viability when PTX concentrations were above 10 nM. For example, cell viability after treatment with 3 nM PTX + 10 ng/mL IFN- γ (26.8%) was significantly lower than that of either 3 nM PTX alone (62%) or 10 ng/mL IFN- γ alone (56.2%, Figure S11B, $p < 0.0001$). These data indicate that IFN- γ was most effective in potentiating the inhibition of cell growth by PTX at low PTX concentrations. This is important since chemotherapeutic drugs have been consistently shown to poorly penetrate into PDAC tissue *in vivo*, resulting in low drug concentrations.³³ Of note, no additive cell killing effect was observed in Panc-1 PDAC cells that had low expression level of IFN- γ receptor at concentrations of PTX ranging from

0.1 to 100 nM (Figure S12), suggesting that the observed additive cell killing effect of PTX and IFN- γ was dependent on IFN- γ signaling.

To investigate the relevance of direct pro-apoptotic actions of IFN- γ in the presence of PTX, we analyzed apoptosis of tumor cells by flow cytometry using Annexin V-FITC/propidium iodide staining (Figure 7D). IFN- γ alone at concentrations from 10 ng/mL to 1000 ng/mL did not have a significant impact on the apoptosis of Kras* cells (Figure S13). The percentage of apoptotic cells was significantly higher after treatment with 10 nM PTX + 3 ng/mL IFN- γ ($54.5 \pm 3.6\%$) than after treatment with 10 nM PTX alone ($38.6 \pm 1.9\%$) or 3 ng/mL IFN- γ alone ($6.4 \pm 0.3\%$) (Figure 7E).

To systematically investigate the signaling pathways affected by treatment of Kras* cells with PTX, IFN- γ , and combined PTX + IFN- γ , we analyzed protein targets in Kras* cells that had significantly changed after each treatment by using reverse-phase protein arrays (Figure S14). Among the 300 targets in the panel, we identified 53 distinct targets that separated the untreated control group from the PTX + IFN- γ group (expression in PTX + IFN- γ group > 1.3 times or < 0.77 times expression in control group); 33 of these targets were downregulated after PTX + IFN- γ treatment, and 20 were upregulated. We then analyzed the changes of target expression using Ingenuity Pathway Analysis. Treatment with PTX + IFN- γ induced the activation of signaling pathways related to IGF1 ($p < 10^{-10}$), ERK/MARK ($p < 10^{-8}$), and apoptosis ($p < 10^{-8}$) and suppression of signaling pathways related to cell cycle (G2/M) ($p < 10^{-8}$) and DNA damage checkpoint regulation ($p < 10^{-6}$). Of note, the pro-apoptosis marker BIM was upregulated in Kras* cells after combined PTX + IFN- γ therapy. BIM levels in cells treated with PTX + IFN- γ increased $42 \pm 5\%$, $15 \pm 3\%$, and $23 \pm 3\%$ compared to un-treated, PTX-treated, and IFN- γ -treated cells, respectively (Figure S14). Elevated BIM expression level was further confirmed by western blot analysis of cell lysates (Figure 7F). These data were consistent with the IHC staining of Kras* tumors from mice treated with combined M-CPA/PTX + anti-PD1, which showed higher expression level of BIM than were observed in tumors after each monotherapy (Figure 7G). BIM is a member of the Bcl-2 family of proteins, which promotes apoptosis. Previously, increased expression level of BIM after PTX treatment was shown to cause apoptosis in breast cancer cells.³⁴ BIM is also a determinant of PTX sensitivity *in vivo*.³⁵ On the other hand, the addition of 1 μ M CPA ($\approx$ intratumoral CPA concentration) to PTX + IFN- γ did not have significant impact on BIM expression level compared to the group of PTX + IFN- γ (Figure S15). Taken together, our data support the idea that the enhanced antitumor efficacy of combined M-CPA/PTX + anti-PD1 was due, at least in part, to increased apoptosis of tumor cells mediated by IFN- γ secreted from tumor-infiltrating CD8⁺ T cells and PTX released from M-CPA/PTX. Our data also indicate that IFN- γ acted in concert with PTX to promote apoptotic cell death, especially in tumor areas where PTX drug concentration was low. Thus, our data provide another rationale (beyond modulation of PDAC stroma to overcome CD8⁺ T-cell exclusion) for combining M-CPA/PTX + anti-PD1 to enhance PDAC cell killing potency.

Combined M-CPA/PTX + anti-PD1 is active in the genetically engineered KPC mouse model of PDAC

Genetically engineered mouse models of PDAC recapitulate the biological and pathophysiological features of the human disease more faithfully than transplantable tumor cell lines, which motivated us to further investigate whether stromal modulation with M-CPA/PTX sensitized PDAC to PD1/PD-L1 blockade in these models. We used KPC-Luc mice bred from LSL-Kras^{G12D/+}; LSL-p53^{T172H/+}; Pdx1-Cre (KPC) mice and *ROSA26-pGAGGs*-LSL-Luciferase mice.³⁶ PDX-1-Cre drives Kras^{G12D} and the inducible luciferase in pancreatic acinar cells, allowing tumor progression to be monitored with bioluminescence imaging. KPC-Luc mice develop spontaneous pancreatic tumors at the age of 100 to 140 days. If left untreated, these mice succumb to extensive local tumor invasion as well as metastasis.²¹ The representative bioluminescence images of KPC-Luc mice from each group are shown in Figure S16, and the survival curves are shown in Figure 8A. The mean survival after enrollment was 18 days for untreated control, 43 days for M-CPA/PTX, 40.5 days for anti-PD1, and 90 days for combined M-CPA/PTX + anti-PD1. All treated groups had significantly longer survival than the untreated control (log-rank test, $p < 0.001$), and the combination therapy group had significantly longer survival than each monotherapy group (log-rank test, $p < 0.05$). We then performed IHC on the tumor tissues collected at one day after the last dose of M-CPA/PTX + anti-PD1 and compared the results with that from the untreated tumors. The frequency of Ki67⁺ nuclei in the M-CPA/PTX + anti-PD1 group was only 25% that in the untreated control group (15 ± 3 vs. 60 ± 4 per visual field, $p < 0.0001$, Figure 8B). The frequency of tumor-infiltrating CD8⁺ T cells in the combination therapy group was 12 times that in untreated control group (25 ± 4 vs. 2 ± 1 per visual field, $p < 0.0001$, Figure 8C). The tumor MVD in the combination therapy group was 2.2 times that in the untreated control group ($2.6 \pm 0.3\%$ vs. $1.2 \pm 0.2\%$, $p < 0.001$, Figure 8D). No difference was observed between these 2 groups in the expression of α -SMA ($9.4 \pm 1.3\%$ vs. $8.8 \pm 1.3\%$, Figure 8E). These data confirmed that combined M-CPA/PTX + anti-PD1 reduced tumor cell proliferation, increased infiltration of CD8⁺ T cells into the tumor bed, and increased blood vessel density without compromising the tumor-restraining stromal components.

CONCLUSIONS

In this study, we investigated whether stromal modulation by M-CPA/PTX could overcome the resistance of PDAC to anti-PD1 therapy. We demonstrated that M-CPA/PTX promoted the infiltration of CD8⁺ T cells. The combination of M-CPA/PTX and anti-PD1 led to significantly higher tumor infiltration by CD8⁺ T cells and higher intratumoral concentration of IFN- γ and consequently significantly prolonged animal survival in an orthotopic murine PDAC model as well as a GEMM of PDAC. We conclude that exclusion of CD8⁺ T cells in PDAC, including the absence of a T-cell infiltrate within the tumor microenvironment, can be partially reversed by stromal modulation pharmacologically with M-CPA/PTX, which increases tumor MVD and reduces hypoxia without depleting collagenous extracellular matrix. Moreover, this study shows that IFN- γ plays a central role in enhanced antitumor activity of a stromal modulation regimen and immune checkpoint inhibition. The key functions of IFN- γ include direct sensitization to PTX-induced apoptotic cell death in

addition to its known roles in enhancing immune surveillance. Increased CD8⁺ T-cell infiltration resulting from combined M-CPA/PTX + anti-PD1 therapy in PDAC appears to be a consequence of dominant effects of stromal modulation with M-CPA/PTX, arguing that strategies for enhancing T-cell tumor infiltration together with ICB therapies will tip the balance in favor of tumor control. Further studies are warranted to understand the molecular mechanisms that underlie the impact of immune infiltrates after M-CPA/PTX therapy, and the role of CCL5 and vascular normalization in enhancing tumor infiltration of CD8⁺ T cells. Such insights should enable the development of therapeutic solutions for T-cell “cold” tumors such as PDAC.

MATERIALS AND METHODS

Preparation and characterization of drug-loaded micelles

The synthesis and characterization of polymers were reported elsewhere.²¹ For preparation of M-CPA/PTX, CPA and PTX, 2.5 mg each, were dissolved in 200 μ L of ethanol. Anionic polymer (100 mg) was dissolved in 0.8 mL of tetrahydrofuran, this solution was added to the drug-ethanol solution, and the mixture was agitated at room temperature for 10 minutes. Cationic polymer (20 mg) was dissolved in 2 mL of deionized water and pipetted into the drug-anionic polymer solution under violent vortexing. The mixture was vortexed for another 1 minute, and then the organic solvents were removed on a rotary evaporator. The micelles were dialyzed against deionized water at 4°C. Un-encapsulated drug was removed by centrifugation. The supernatant was collected and filtered through a sterile 0.22- μ m nylon filter before use. For long-term storage, the micelles were mixed with sucrose solution (50% by weight in water) at a volume ratio of 9:1 and then frozen in a -80°C freezer.

The detailed characterization of M-CPA/PTX, including drug loading, size, surface charge, and drug release, were reported elsewhere.²¹ The morphology of M-CPA/PTX was examined by transmission electron microscopy (TEM). Samples were placed on 100 mesh formvar-coated copper grids treated with poly-L-lysine and left for 1 hour. The grids were blotted with filter paper to remove excess samples and then stained with filtered 1% uranyl acetate for 1 minute. Stain was blotted from the grids with filter paper, and the samples were allowed to dry. TEM was performed on a JEM 1010 transmission electron microscope (JEOL USA, Inc., Peabody, MA) at an accelerating voltage of 80 kV. Digital images were collected using the AMT Imaging System (Advanced Microscopy Techniques Corp., Danvers, MA).

Cell lines and animals

All animal studies were approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center and were carried out in accordance with institutional guidelines. The Kras^{*} orthotopic murine PDAC model was established by injecting Kras^{*} PDAC cells²² into the pancreas of 6-week-old female C57BL/6 mice (Taconic Biosciences, Rensselaer, NY). A small incision was made in the left abdomen of each mouse, and then 0.5 million Kras^{*} cells in 10 μ L of Hank's balanced salt solution were injected in the pancreas. The incision was closed with absorbable sutures, and the tumors were allowed to grow to about 5 mm in diameter before treatment.

KPC-Luc mice were generated by breeding LSL-Kras^{G12D/+}; LSL-p53^{T172H/+}; Pdx1-Cre (KPC) mice³⁷ with *ROSA26-pGAGGs*-LSL-Luciferase mice³⁶. Bioluminescence imaging (Xenogen IVIS-200 optical system, Perkin Elmer, Waltham, MA) was used to monitor tumor presence in KPC-Luc mice. Mice were anesthetized with 3% isoflurane and intraperitoneally injected with D-Luciferin (Perkin Elmer, Waltham, MA) at 150 mg/kg body weight. Images were acquired every 2 minutes from 4 to 20 minutes after the injection of D-luciferin using Living Imaging analysis and acquisition software (Xenogen), and the highest value of integrated photon flux was recorded as an index for tumor burden. A photographic image was also taken and projected with the pseudo-color image representing the spatial distribution of photon counts.

In vivo antitumor efficacy

For the Kras* orthotopic murine PDAC model, mice were enrolled once tumor reached 5 mm in diameter. M-CPA/PTX was intravenously injected at 5 mg/kg/dose for 3 consecutive days. Anti-PD1 was intraperitoneally injected at 100 µg/mouse every other day starting 48 hours after the last dose of M-CPA/PTX. For the KPC-Luc model, treatment was initiated once tumor became palpable in mice at 120 to 130 days of age after positive bioluminescence imaging. Mice were first injected intravenously with M-CPA/PTX at 5 mg/kg/dose for 6 doses over 2 weeks. Anti-PD1 was intraperitoneally injected at 100 µg/mouse every other day for 6 doses starting 48 hours after the last dose of M-CPA/PTX.

Cell proliferation and apoptosis

To measure proliferation, cells were seeded at 1000 cells/well in 96-well plates and treated at 37°C for 72 hours. Cells were then stained with crystal violet, and the absorption at 570 nm was measured to quantify cell numbers. The proportion of cells was normalized to that of untreated cells and expressed as mean ± standard error of the mean (SEM) (n = 6). For apoptosis analysis, cells were seeded at 1 × 10⁵ cells per well in 6-well plates overnight and treated for 48 hours at 37°C. At the end of treatments, cells were detached and stained with Annexin V-FITC/propidium iodide (Molecular Probes Inc., OR) and analyzed with flow cytometry (BD FACSCalibur, CA). Untreated cells were used as controls.

Tumor digestion

Weighed tumors were minced and digested in an 8-ml mixture of 2 mg/ml collagenase type IV (LS004188, Worthington, NJ), 0.2 mg/ml hyaluronidase (H3506, Sigma, MO), and 0.2 mg/ml DNase I (D4527, Sigma) in DMEM/F12 medium at 37°C for 1 hour. The mixture was constantly shaken at 20 RPM. Debris was removed by filtration through a 40-µm mesh, and the red blood cells were removed using a red blood cell lysis buffer (R7757, Sigma, MO). The mixture was pelleted and re-suspended in 5 ml of PBS and slowly added on top of 5-ml Lympholyte-M (Cedarlane Labs, NY) in a 15-ml conical tube. The tubes were then centrifuged for 20 minutes at 1,200 g at room temperature with both acceleration and break to the lowest ramps. After centrifuge, cells between the PBS and Lympholyte-M layers was carefully transferred to a new conical tube, pelleted, and washed with PBS before further analyses.

Analyses of immune cells

Red blood cell lysis was performed for spleens. Cells were stained for Live/Dead Fixable Aqua Dead Cell Stain Kit (Invitrogen, MA) and then incubated with Fc-block (BD Pharmingen, CA). Cells were stained with antibodies against CD11b (clone M1/79), Ly6C (clone AL-21), Ly6G (clone 1A8), CD19 (clone 1D3), CD8a (clone 53-6.7), PD1 (clone J43), and TIGIT (clone 1G9) from BD Pharmingen, CA; F4/80 (clone BM8), PD-L1 (clone 10F.9G2), CD45 (clone 30-F11), CD4 (clone GK1.5), and NK1.1 (clone PK136) from BioLegend, CA; and CD11c (clone N418), major histocompatibility complex class II (I-A/I-E, clone M5/114.15.2), Foxp3 (clone FJK.16s), and CTLA-4 (clone 9H10) from eBioscience, CA. Intracellular staining was performed after fixation and permeabilization with Foxp3/Transcription Factor Fixation/Permeabilization kit (eBioscience, CA). Samples were acquired on a BD Fortessa cytometer (X-20, BD Biosciences, CA) and analyzed with FlowJo (10.0.7) software.

Cytokine/chemokine assay

Kras⁺ PDAC tumors retrieved on day 10 after the initiation of treatments were mechanically disrupted. Supernatant was collected after centrifuging. Cytokines/chemokines were measured using a Milliplex mouse cytokine/chemokine panel (Millipore Sigma, WI) according to the manufacturer's protocol. Fluorescence signal was measured on a Luminex 100/200 system (Luminexcorp, TX), and data were analyzed using GraphPad software.

Western blotting

Cell or tumor lysate was fractioned on NUPAGE 4% to 12% Bis-Tris gels (Thermo Fisher Scientific, TX) and transferred to a poly(vinylidene fluoride) membrane (Millipore, MA). Membranes were blocked with Odyssey blocking buffer (LI-COR, NE), blotted with the primary antibodies listed in Supplemental Table S1, and visualized using fluorophore-conjugated anti-rabbit or anti-goat IgGs (LI-COR, Lincoln, NE) on an Odyssey near-infrared fluorescence scanner (LI-COR, NE).

Quantitative RT-PCR

RNA was isolated from homogenized snap-frozen tumor tissues or cultured cells using the Direct-zol kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions. cDNA was transcribed from RNA using a Verso cDNA synthesis kit (Thermo Fisher Scientific) according to the manufacturer's instructions. qRT-PCR was done using Power SYBR Green PCR master mix (Applied Biosystems, Thermo Fisher Scientific) with the primers listed in Supplemental Table S2 on a Bio-Rad CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA). Relative gene expression was calculated using the 2^{-Ct} method and normalized to endogenous controls.

Immunohistochemical staining

Tumors were harvested, fixed in formalin, and embedded in paraffin before being cut into 4- μ m sections. Sections for IHC were de-paraffinized, rehydrated, and subjected to antigen retrieval for 30 minutes in 95°C 10-mM citrate buffer (pH 6). After antigen retrieval, slides were blocked in Tris-buffered saline (pH 7.4) supplemented with 0.1% tween-20 and 10%

goat or donkey serum prior to incubation with primary antibodies (Supplemental Table S1) overnight at 4°C. To visualize staining using the 3,3'-diaminebenzidine (DAB) system, slides were washed and incubated with biotinylated anti-rabbit or anti-goat IgG (Vector Labs, CA) and streptavidin-conjugated horseradish peroxidase (DAKO, CA) for 30 minutes each. A positive reaction was detected by exposure to DAB according to the manufacturer's instructions. Slides were counterstained with hematoxylin and visualized under a bright-field microscope at 100× or 200× magnification. For fluorescence visualization, slides were incubated with fluorophore-conjugated anti-rabbit or anti-goat IgGs (Cell Signaling, MA) at room temperature for 1 hour and counterstained with Hoechst 33342. Slides were visualized using a Zeiss Axio Observer.Z1 fluorescence microscope. Staining was quantified using at least 10 randomly selected 20× fields of view. Control and treated mice within the same experimental set (at least 5 mice per group) were analyzed. All staining was quantified using NIH Image J analysis software (<http://rsb.info.nih.gov/ni-image/>) with the same threshold for each stain.

UPLC-MS/MS assay for simultaneous quantification of CPA and PTX

Detailed procedures are described in our previous publication.²¹ Briefly, tumors were homogenized for quantification of PTX and CPA, and the chromatographic separation was achieved on Hypersil GOLD C18 Selectivity LC Columns (1.9 μm, 50 × 2.1 mm, Thermo Fisher Scientific, Madison, WI) with a gradient elution using mobile phase A of 0.1% formic acid in water and mobile phase B of 0.1% formic acid in acetonitrile. The detection was performed with a triple quadrupole mass spectrometer (API 5500, Applied Biosystems/MDS SCIEX, Foster City, CA) in the positive ionization using APCI source and multiple reaction monitoring mode, using docetaxel as an internal standard for quantification. Linearity of the assay was demonstrated over the range of 0.5 ng/ml to 2,000 ng/ml, with the lower limit of quantification of 0.5 ng/ml for both PTX and CPA, in mouse blood and pancreatic tumor. The assay was accurate and precise with bias and percentage in coefficient of variation of less than 15% and extraction recovery of 85% to 104% for PTX and 85% to 89% for CPA.

RPPA analysis

The reverse phase protein array (RPPA) analysis was performed in the Functional Proteomics RPPA Core Facility at the University of Texas MD Anderson Cancer Center. Kras* cell lysate was prepared using RPPA lysis buffer, manually serial-diluted in five 2-fold dilutions with the lysis buffer, and printed on nitrocellulose-coated slides using an Aushon Biosystem 2470 arrayer (Billerica, MA). The slides were then probed with approximately 300 validated primary antibodies, followed by detection with corresponding biotinylated secondary antibodies. The signals obtained was amplified using a Cytomation-catalyzed system of Avidin-biotinylated peroxidase (Vectastain Elite ABC kit from Vector Lab, Burlingame, CA) binding to the secondary antibodies, and catalyzing the conjugation of Tyramide-biotin to form insoluble biotinylated phenols. Signals were visualized by a secondary streptavidin-conjugated HRP and DAB colorimetric reaction. The slides were scanned and quantified using Array-Pro Analyzer software (Media Cybernetics, Rockville, MD) to generate spot intensity (Level 1 data). SuperCurve GUI was used to estimate relative protein levels (in log2 scale). A fitted curve ("supercurve") was created with signal

intensities on the Y-axis and relative log2 amounts of each protein on the X-axis using a non-parametric, monotone increasing B-spline model. Raw spot intensity data were adjusted to correct spatial bias before model fitting using “control spots” arrayed across the slides. A QC metric was generated for each slide to determine slide quality and only slides with 0.8 on a 0–1 scale were used further. For replicate slides, the slide with the highest QC score was used for analysis (Level 2 data). Protein measurements were corrected for loading using median-centering across antibodies (Level 3 data). Samples with low protein levels were excluded from further analysis.

For statistical analysis, clustering analysis was first performed on the targets, using Wald’s linkage hierarchical clustering of the Spearman correlation of samples. The samples were ordered by their treatment group. The FC (fold change) values were calculated as the estimated ratio between the 2 groups in comparison, with the following conventional modification: For the ratios > 1 (up-regulation), FCs were noted as the same as the ratio. For the ratios < 1 (down-regulation), FC were noted as the negative inverse of the ratio. The absolute value of fold change 1.3 was used as the cut off value of significant proteins in the comparisons of treatment groups. Heat maps were prepared for each comparison. The data were scaled for each gene across samples only for heat maps.

Statistics

Values are expressed as mean $\pm$ SEM. Data were evaluated using Student’s t test or one-way analysis of variance (ANOVA) followed by post hoc Tukey multiple comparisons. The log-rank test was used in Kaplan-Meier survival analyses. A p value of less than 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Tempero MA; Berlin J; Ducreux M; Haller D; Harper P; Khayat D; Schmoll HJ; Sobrero A; Van Cutsem E Pancreatic Cancer Treatment and Research: an International Expert Panel Discussion. *Ann. Oncol* 2011, 22, 1500–1506. [PubMed: 21199884]
2. Wojtowicz ME; Dunn BK; Umar A Immunologic Approaches to Cancer Prevention-Current Status, Challenges, and Future Perspectives. *Semin. Oncol* 2016, 43, 161–172. [PubMed: 26970135]
3. Thind K; Padmos LJ; Ramanathan RK; Borad MJ Immunotherapy in Pancreatic Cancer Treatment: a New Frontier. *Therap. Adv. Gastroenterol* 2017, 10, 168–194.
4. Pardoll DM The Blockade of Immune Checkpoints in Cancer Immunotherapy. *Nat. Rev. Cancer* 2012, 12, 252–264. [PubMed: 22437870]
5. Topalian SL; Hodi FS; Brahmer JR; Gettinger SN; Smith DC; McDermott DF; Powderly JD; Carvajal RD; Sosman JA; Atkins MB; Leming PD; Spigel DR; Antonia SJ; Horn L; Drake CG;

- Pardoll DM; Chen L; Sharfman WH; Anders RA; Taube JM; et al. Safety, Activity, and Immune Correlates of Anti-PD-1 Antibody in Cancer. *N. Engl. J. Med* 2012, 366, 2443–2454. [PubMed: 22658127]
6. Ji RR; Chasalow SD; Wang L; Hamid O; Schmidt H; Cogswell J; Alaparthi S; Berman D; Jure-Kunkel M; Siemers NO; Jackson JR; Shahabi V An Immune-active Tumor Microenvironment Favors Clinical Response to Ipilimumab. *Cancer Immunol. Immunother* 2012, 61, 1019–1031. [PubMed: 22146893]
 7. Cipponi A; Wieers G; van Baren N; Coulie PG Tumor-infiltrating Lymphocytes: Apparently Good for Melanoma Patients. But Why? *Cancer Immunol. Immunother* 2011, 60, 1153–1160. [PubMed: 21553145]
 8. Feig C; Gopinathan A; Neesse A; Chan DS; Cook N; Tuveson DA The Pancreas Cancer Microenvironment. *Clin. Cancer Res* 2012, 18, 4266–4276. [PubMed: 22896693]
 9. Martinez-Bosch N; Vinaixa J; Navarro P Immune Evasion in Pancreatic Cancer: from Mechanisms to Therapy. *Cancers (Basel)* 2018, 10, 6.
 10. Feig C; Jones JO; Kraman M; Wells RJ; Deonaraine A; Chan DS; Connell CM; Roberts EW; Zhao Q; Caballero OL; Teichmann SA; Janowitz T; Jodrell DI; Tuveson DA; Fearon DT Targeting CXCL12 from FAP-expressing Carcinoma-associated Fibroblasts Synergizes with Anti-PD-L1 Immunotherapy in Pancreatic Cancer. *Proc. Natl. Acad. Sci. U. S. A* 2013, 110, 20212–20217. [PubMed: 24277834]
 11. Ozdemir BC; Pentcheva-Hoang T; Carstens JL; Zheng X; Wu CC; Simpson TR; Laklai H; Sugimoto H; Kahlert C; Novitskiy SV; De Jesus-Acosta A; Sharma P; Heidari P; Mahmood U; Chin L; Moses HL; Weaver VM; Maitra A; Allison JP; LeBleu VS; et al. Depletion of Carcinoma-associated Fibroblasts and Fibrosis Induces Immunosuppression and Accelerates Pancreas Cancer with Reduced Survival. *Cancer Cell* 2014, 25, 719–734. [PubMed: 24856586]
 12. Lee JJ; Perera RM; Wang H; Wu DC; Liu XS; Han S; Fitamant J; Jones PD; Ghanta KS; Kawano S; Nagle JM; Deshpande V; Boucher Y; Kato T; Chen JK; Willmann JK; Bardeesy N; Beachy PA Stromal Response to Hedgehog Signaling Restrains Pancreatic Cancer Progression. *Proc. Natl. Acad. Sci. U. S. A* 2014, 111, E3091–3100. [PubMed: 25024225]
 13. Provenzano PP; Cuevas C; Chang AE; Goel VK; Von Hoff DD; Hingorani SR Enzymatic Targeting of the Stroma Ablates Physical Barriers to Treatment of Pancreatic Ductal Adenocarcinoma. *Cancer Cell* 2012, 21, 418–429. [PubMed: 22439937]
 14. Guo S; Contratto M; Miller G; Leichman L; Wu J Immunotherapy in Pancreatic Cancer: Unleash Its Potential through Novel Combinations. *World J. Clin. Oncol* 2017, 8, 230–240. [PubMed: 28638792]
 15. Hingorani SR; Harris WP; Beck JT; Berdov BA; Wagner SA; Pshevlotsky EM; Tjulandin SA; Gladkov OA; Holcombe RF; Korn R; Raghunand N; Dychter S; Jiang P; Shepard HM; Devoe CE Phase Ib Study of PEGylated Recombinant Human Hyaluronidase and Gemcitabine in Patients with Advanced Pancreatic Cancer. *Clin. Cancer Res* 2016, 22, 2848–2854. [PubMed: 26813359]
 16. Yauch RL; Gould SE; Scales SJ; Tang T; Tian H; Ahn CP; Marshall D; Fu L; Januario T; Kallop D; Nannini-Pepe M; Kotkow K; Marsters JC; Rubin LL; de Sauvage FJ A Paracrine Requirement for Hedgehog Signalling in Cancer. *Nature* 2008, 455, 406–410. [PubMed: 18754008]
 17. Onishi H; Katano M Hedgehog Signaling Pathway as a New Therapeutic Target in Pancreatic Cancer. *World J. Gastroenterol* 2014, 20, 2335–2342. [PubMed: 24605030]
 18. Rhim Andrew D.; Oberstein Paul E.; Thomas Dafydd H.; Mirek Emily T.; Palermo Carmine F.; Sastra Stephen A.; Dekleva Erin N.; Saunders T; Becerra Claudia P.; Tattersall Ian W.; Westphalen CB; Kitajewski J; Fernandez-Barrena Maite G.; Fernandez-Zapico Martin E.; Iacobuzio-Donahue C; Olive Kenneth P.; Stanger Ben Z. Stromal Elements Act to Restrain, rather than Support, Pancreatic Ductal Adenocarcinoma. *Cancer Cell* 2014, 25, 735–747. [PubMed: 24856585]
 19. De Jesus-Acosta A; O'Dwyer PJ; Ramanathan RK; Von Hoff DD; Maitra A; Rasheed Z; Zheng L; Rajeshkumar NV; Le DT; Hoering A; Bolejack V; Yabuuchi S; Laheru DA A phase II Study of Vismodegib, a Hedgehog (Hh) Pathway Inhibitor, Combined with Gemcitabine and Nab-paclitaxel (nab-P) in Patients (pts) with Untreated Metastatic Pancreatic Ductal Adenocarcinoma (PDA). *J. Clin. Oncol* 2014, 32 (Suppl 3; Abstr 257).
 20. Catenacci DV; Junttila MR; Karrison T; Bahary N; Horiba MN; Nattam SR; Marsh R; Wallace J; Kozloff M; Rajdev L; Cohen D; Wade J; Sleckman B; Lenz HJ; Stiff P; Kumar P; Xu P;

- Henderson L; Takebe N; Salgia R; et al. Randomized Phase IB/II Study of Gemcitabine plus Placebo or Vismodegib, a Hedgehog Pathway Inhibitor, in Patients with Metastatic Pancreatic Cancer. *J. Clin. Oncol* 2015, 33, 4284–4292. [PubMed: 26527777]
21. Zhao J; Wang H; Hsiao CH; Chow DS; Koay EJ; Kang Y; Wen X; Huang Q; Ma Y; Bankson JA; Ullrich SE; Overwijk W; Maitra A; Piwnica-Worms D; Fleming JB; Li C Simultaneous Inhibition of Hedgehog Signaling and Tumor Proliferation Remodels Stroma and Enhances Pancreatic Cancer Therapy. *Biomaterials* 2018, 159, 215–228. [PubMed: 29331808]
 22. Ying H; Kimmelman AC; Lyssiotis CA; Hua S; Chu GC; Fletcher-Sananikone E; Locasale JW; Son J; Zhang H; Coloff JL; Yan H; Wang W; Chen S; Viale A; Zheng H; Paik JH; Lim C; Guimaraes AR; Martin ES; Chang J; et al. Oncogenic Kras Maintains Pancreatic Tumors through Regulation of Anabolic Glucose Metabolism. *Cell* 2012, 149, 656–670. [PubMed: 22541435]
 23. Olsson AK; Dimberg A; Kreuger J; Claesson-Welsh L VEGF Receptor Signalling - in Control of Vascular Function. *Nat. Rev. Mol. Cell Biol* 2006, 7, 359–371. [PubMed: 16633338]
 24. Eliceiri BP; Paul R; Schwartzberg PL; Hood JD; Leng J; Cheresh DA Selective Requirement for Src Kinases during VEGF-induced Angiogenesis and Vascular Permeability. *Mol. Cell* 1999, 4, 915–924. [PubMed: 10635317]
 25. Issbrucker K; Marti HH; Hippenstiel S; Springmann G; Voswinckel R; Gaumann A; Breier G; Drexler HC; Suttrop N; Clauss M p38 MAP Kinase--a Molecular Switch between VEGF-induced Angiogenesis and Vascular Hyperpermeability. *FASEB J* 2003, 17, 262–264. [PubMed: 12490545]
 26. Joyce JA; Fearon DT T Cell Exclusion, Immune Privilege, and the Tumor Microenvironment. *Science* 2015, 348, 74–80. [PubMed: 25838376]
 27. Chiu DK; Tse AP; Xu IM; Di Cui J; Lai RK; Li LL; Koh HY; Tsang FH; Wei LL; Wong CM; Ng IO; Wong CC Hypoxia Inducible Factor HIF-1 Promotes Myeloid-Derived Suppressor Cells Accumulation through ENTPD2/CD39L1 in Hepatocellular Carcinoma. *Nat. Commun* 2017, 8, 517. [PubMed: 28894087]
 28. Bellucci R; Martin A; Bommarito D; Wang K; Hansen SH; Freeman GJ; Ritz J Interferon-gamma-induced Activation of JAK1 and JAK2 Suppresses Tumor Cell Susceptibility to NK cells through Upregulation of PD-L1 Expression. *Oncoimmunology* 2015, 4, e1008824. [PubMed: 26155422]
 29. Winograd R; Byrne KT; Evans RA; Odorizzi PM; Meyer AR; Bajor DL; Clendenin C; Stanger BZ; Furth EE; Wherry EJ; Vonderheide RH Induction of T-cell Immunity Overcomes Complete Resistance to PD-1 and CTLA-4 Blockade and Improves Survival in Pancreatic Carcinoma. *Cancer Immunol. Res* 2015, 3, 399–411. [PubMed: 25678581]
 30. Zhong H; Han B; Tourkova IL; Lokshin A; Rosenbloom A; Shurin MR; Shurin GV Low-dose Paclitaxel Prior to Intratumoral Dendritic Cell Vaccine Modulates Intratumoral Cytokine Network and Lung Cancer Growth. *Clin. Cancer Res* 2007, 13, 5455–5462. [PubMed: 17875775]
 31. Spranger S; Bao R; Gajewski TF Melanoma-intrinsic beta-Catenin Signalling Prevents Anti-tumour Immunity. *Nature* 2015, 523, 231–235. [PubMed: 25970248]
 32. Harlin H; Meng Y; Peterson AC; Zha Y; Tretiakova M; Slingluff C; McKee M; Gajewski TF Chemokine Expression in Melanoma Metastases Associated with CD8+ T-cell Recruitment. *Cancer Res* 2009, 69, 3077–3085. [PubMed: 19293190]
 33. Michl P; Gress TM Improving Drug Delivery to Pancreatic Cancer: Breaching the Stromal Fortress by Targeting Hyaluronic Acid. *Gut* 2012, 61, 1377–1379. [PubMed: 22661496]
 34. Sunter A; Fernandez de Mattos S; Stahl M; Brosens JJ; Zoumpoulidou G; Saunders CA; Coffey PJ; Medema RH; Coombes RC; Lam EW FoxO3a Transcriptional Regulation of Bim Controls Apoptosis in Paclitaxel-treated Breast Cancer Cell Lines. *J. Biol. Chem* 2003, 278, 49795–49805. [PubMed: 14527951]
 35. Tan TT; Degenhardt K; Nelson DA; Beaudoin B; Nieves-Neira W; Bouillet P; Villunger A; Adams JM; White E Key Roles of BIM-driven Apoptosis in Epithelial Tumors and Rational Chemotherapy. *Cancer Cell* 2005, 7, 227–238. [PubMed: 15766661]
 36. Safran M; Kim WY; Kung AL; Horner JW; DePinho RA; Kaelin WG, Jr. Mouse Reporter Strain for Noninvasive Bioluminescent Imaging of Cells that Have Undergone Cre-mediated Recombination. *Mol. Imaging* 2003, 2, 297–302. [PubMed: 14717328]
 37. Olive KP; Jacobetz MA; Davidson CJ; Gopinathan A; McIntyre D; Honess D; Madhu B; Goldgraben MA; Caldwell ME; Allard D; Frese KK; Denicola G; Feig C; Combs C; Winter SP;

Ireland-Zecchini H; Reichelt S; Howat WJ; Chang A; Dhara M; et al. Inhibition of Hedgehog Signaling Enhances Delivery of Chemotherapy in a Mouse Model of Pancreatic Cancer. *Science* 2009, 324, 1457–1461. [PubMed: 19460966]

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Figure 1.

Schematic illustration of M-CPA/PTX nanoformulation. M-CPA/PTX was self-assembled by mixing anionic and cationic block copolymers in the presence of CPA and PTX. The resultant M-CPA/PTX was spherical and between 50 and 100 nm in diameter, as shown in the transmission electron micrograph.

Figure 2.

M-CPA/PTX modulated Kras* tumor-associated stroma in favor of immunotherapy. (A) Schedule for treatment and tissue collection. Mice were enrolled once tumors were palpable, and intravenously injected with M-CPA/PTX at 5 mg/kg/drug daily on days 0, 1, and 2. Tumor tissues were collected on days 2, 4, and 6 after the initiation of treatment. (B) Representative micrographs of CD31 (green) immunofluorescence staining and corresponding quantifications. Ten visual fields were randomly captured for each group. Scale bars = 50 μ m. (C) Immunoblotting of vascular endothelial growth factor (VEGF) signaling (N = 3). FAK: focal adhesion kinase. PLC γ 1: phosphoinositide-specific phospholipase C- γ 1. Src: Src family of protein tyrosine kinase. AKT: protein kinase B. Erk: extracellular signal-regulated kinase. MAPK: Mitogen-activated protein kinase. (D)

Immunoblotting of Shh ligand, carbonic anhydrase 9 (CA-IX), and FAP- α (N = 3 each group). Data are presented as mean $\pm$ SEM. Significance was determined using 1-way ANOVA followed by Tukey post hoc analysis. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, FOV = field of view, ns = not significant.

Figure 3. Combination of M-CPA/PTX and anti-PD1 extended animal survival in Kras* model. (A) Treatment schedule. (B) Kaplan-Meier survival analysis of tumor-bearing mice (N = 10). ****p < 0.0001, log-rank test. Kras* murine pancreatic cancer cells were inoculated in the pancreas. Mice were enrolled once tumor was palpable.

Figure 4.

Treatment effects on tumor and stromal compartments. (A) Schedule of treatment and tumor collection for the short-term response study. (B) Mouse body weight. (C) Tumor weight. Ten mice were included in each group. (D-G) Representative micrographs of Ki67 (D), CD31 (E), Picrosirius Red (F), and α-SMA (G) IHC staining and corresponding quantifications. Scale bar = 50 μm for panels D, E, and G; scale bar = 100 μm for panel F. Ten to fifteen visual fields were randomly captured for each group. Data are presented as mean ± SEM. Significance was determined using 1-way ANOVA followed by Tukey post hoc analysis. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, FOV = field of view, ns = not significant.

Figure 5.

Analyses of intratumoral immune cells and cytokines after treatment. (A-E) Quantification of intratumoral immune cells per gram of tumor (10 tumors per group): (A) CD8⁺ T cells (CD45⁺CD8⁺); (B) Regulatory T cells (Treg, CD45⁺CD4⁺CD25⁺Foxp3⁺); (C) Myeloid-derived suppressive cells (MDSC, CD45⁺CD11b⁺CD11c⁻Ly6G⁺ or CD45⁺CD11b⁺CD11c⁻Ly6C⁺); (D) Macrophages (CD45⁺CD11b⁺F4/80⁺); (E) Dendritic cells (CD45⁺CD11b⁺CD11c⁺). (F&G) Quantification of cytokines per gram of tumor (5 tumors per group): IFN-γ (F) and CCL5 (G). Tumor-bearing mice were enrolled for treatment once tumors became palpable. Data are presented as mean ± SEM. Significance was determined using 1-way ANOVA followed by Tukey post hoc analysis. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns = not significant.

Figure 6.

Effects of CD8α⁺ T cells and IFN-γ on antitumor efficacy. (A) Kaplan-Meier survival analysis of tumor-bearing mice with and without depletion of CD8⁺ T cells (N = 10 per group). ****p < 0.0001, log-rank test. (B) Tumor growth curves with and without neutralization of IFN-γ (N = 10 per group). (C, D) Representative micrographs of tumor-penetrating CD8α⁺ T cells (C) and IFN-γ (D) staining and corresponding quantifications. Ten to fifteen visual fields were randomly captured for each group. Scale bars = 50 μm. Data are presented as mean ± SEM. Significance was determined using 1-way ANOVA followed by Tukey post hoc analysis. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns = not significant.

Figure 7.

Effect of combined PTX and IFN-γ. (A-C) Kras* cell proliferation in the presence of different concentrations of IFN-γ (A), PTX (B), and PTX alone or with IFN-γ (C). N = 6 per group. (D, E) Representative flow cytometry scatter plots (D) and quantification (E) of Kras* cell apoptosis induced by PTX and/or IFN-γ. N = 3 per group. (F) Western blot validation of BIM in Kras* cell culture. (G) IHC validation of BIM in Kras* orthotopic tumor. Scale bar = 50 μm. Data are presented as mean ± SEM. Significance was determined using 1-way ANOVA followed by Tukey post hoc analysis. **p < 0.01, ****p < 0.0001.

Figure 8.

Antitumor efficacy in KPC-Luc mice. (A) Kaplan-Meier survival analysis. N = 10 per group.

* $p < 0.05$, log-rank test. KPC-Luc mice were enrolled at the age of 120 to 130 days once tumor became palpable. (B-E) Representative micrographs of Ki67⁺ nuclei (B), tumor-penetrating CD8 α ⁺ T cells (C), CD31 (D), and α -SMA (E), and corresponding quantifications. Tumors were collected 1 day after the last dose of treatment. Fifteen visual fields were randomly captured for each group. Scale bars = 50 μ m. Data are presented as

mean $\pm$ SEM. Significance was determined using Student's *t* test. ****p* < 0.001, *****p* < 0.0001, ns, not significant.

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Table 1.Long-term stability of M-CPA/PTX^a

M-CPA/PTX Characteristic	Day 0	Day 90
Diameter, nm ^b	61.5 ± 1.9	53.2 ± 3.2
Polydispersity index	0.131 ± 0.04	0.181 ± 0.12
Relative CPA loading, %	100 ± 3.1	99.4 ± 1.7
Relative PTX loading, %	100 ± 2.6	100.5 ± 3.5

SD, standard deviation.

^a Micelles were snap-frozen in the presence of 5% sucrose. Data are presented as mean ± standard deviation (n = 3). CPA and PTX were equally loaded in micelles at 2.5% by weight for each drug.

^b Number-average size determined by dynamic light scattering.