



Deposited via The University of Sheffield.

White Rose Research Online URL for this paper:

<https://eprints.whiterose.ac.uk/id/eprint/245002/>

Version: Published Version

---

**Article:**

Smith, J.P., Mohit, M., Kenner, J. et al. (2026) Interruption of cross-communication pathways alters the immune cell signature of pancreatic cancer and decreases tumor growth. *Cell Communication and Signaling*, 24 (1). 71. ISSN: 1478-811X

<https://doi.org/10.1186/s12964-026-02676-8>

---

**Reuse**

This article is distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND) licence. This licence only allows you to download this work and share it with others as long as you credit the authors, but you can't change the article in any way or use it commercially. More information and the full terms of the licence here: <https://creativecommons.org/licenses/>

**Takedown**

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing [eprints@whiterose.ac.uk](mailto:eprints@whiterose.ac.uk) including the URL of the record and the reason for the withdrawal request.

RESEARCH

Open Access



# Interruption of cross-communication pathways alters the immune cell signature of pancreatic cancer and decreases tumor growth

Jill P. Smith<sup>1\*</sup>, Mahdis Mohit<sup>2</sup>, Joi Kenner<sup>3</sup>, Ajay Nathan<sup>1</sup>, Jack Drda<sup>4</sup>, Martha D. Gay<sup>1</sup>, Hong Cao<sup>1</sup>, Wenqiang Chen<sup>1</sup>, Alison Gomez<sup>5</sup>, Elisa Baldelli<sup>5</sup>, Wenyu Dou<sup>6</sup>, Hong-Bin Fang<sup>6</sup>, Narayan Shivapurkar<sup>1</sup>, James Davis<sup>5</sup>, Qi Wei<sup>7</sup>, Abdellah Akhrif<sup>7</sup> and Mariaelena Pierobon<sup>5</sup>

## Abstract

**Background** Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy with limited therapeutic options in part due to a dense stroma and the ability of the tumor cells to defy therapy by creating an immunosuppressive milieu. A histologic feature of PDAC is the abundance of immunosuppressive M2-polarized tumor-associated macrophages (TAMs) that favor cancer growth and metastases. Cancer cells communicate with immune cells of the tumor microenvironment (TME) by several mechanisms including exosomes to induce plasticity of the immune cells favoring pro-cancer instead of anti-cancer phenotypes. We examined a novel strategy to interrupt the cross-communication between cancer and immune cells by reprogramming the TME with a cholecystikinin-B receptor (CCK-BR) antagonist, proglumide.

**Methods** Cross-communication was examined using 3-dimensional human PDAC spheroids that were either co-cultured with macrophages or treated with supernatant spent media from macrophages. RNA was extracted from the spheroids and subjected to RNA sequencing and Reverse Phase Protein Array (RPPA). RNA sequencing revealed that the most upregulated gene in the cancer cells after co-culture was *ELANE*, which codes for neutrophil elastase. Next, *ELANE* expression was down regulated by shRNA transfection in PDAC cells and the effect of the decreased expression was examined on the polarization of immune cells *in vitro* and *in vivo* in mice. Exosomes were also collected from PDAC spheroids for evaluation by RPPA.

**Results** Co-culture of spheroids with macrophages increased cancer cell number and induced polarization of M0 macrophages to the M2-phenotype. Co-culture increased oncogenic pathways and genes by RPPA and RNA sequencing, respectively, and these changes were reversed with proglumide. *ELANE* knockdown in PDAC cells prevented the M2-polarization of macrophages *in vitro* and decreased tumor growth *in vivo*. Immunohistochemistry of *ELANE* knockdown tumors showed a shift in plasticity from M2-polarized TAMs to M1-polarized cells compared to

\*Correspondence:

Jill P. Smith  
jps261@georgetown.edu

Full list of author information is available at the end of the article



© The Author(s) 2026. **Open Access** This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

wild-type tumors. Neutrophil elastase in cancer exosomes increased after co-culture and was abolished by treatment with proglumide.

**Conclusion** *ELANE* expression in PDAC cells regulates polarization of macrophages through the release of neutrophil-elastase rich exosomes. Proglumide therapy with CCK-B receptor antagonism is a novel method to interrupt cross-communication pathways between PDAC and immune cells in the TME and decrease cancer growth.

**Keywords** Tumor microenvironment, Cell signaling, Cytokines, Neutrophil elastase, Tumor-associated macrophages, Pancreatic cancer

## Introduction

Pancreatic ductal adenocarcinoma (PDAC) has a complex tumor microenvironment (TME) consisting of various cellular and non-cellular components that interact in intricate ways, contributing to the aggressive nature of this cancer. Interactions among components in TME not only support tumor growth and spread but also create a formidable barrier to effective therapeutic interventions, making PDAC notoriously difficult to treat [1]. In the PDAC TME, there is an abundance of M2-polarized macrophages, which are pro-tumorigenic and play a crucial role in promoting tumor growth and metastasis by suppressing anti-tumor immune responses and facilitating tissue remodeling and angiogenesis. PDAC tumor progression is driven by the communication between the cancer epithelial cells and the surrounding cells in the microenvironment. Communication can be mediated by direct cell–cell contact, by the release of secretory proteins such as chemokines, cytokines, and growth factors, and through the release of extracellular vesicles (EVs) [2] that are lipid bilayer particles secreted by cells into the extracellular microenvironment. EVs released from epithelial cancer cells contain various types of biomolecules, such as proteins, RNA, microRNAs, and lipids, which can modulate the function of neighboring cells, including macrophages [3]. PDAC-derived EVs can regulate angiogenesis, tumor growth, and immune regulation of the TME [2, 4, 5].

Immune cells in the TME often communicate and recruit other cells by releasing chemokines. These inflammatory mediators activate chemokine receptors that are G-protein-coupled receptors (GPCRs). M2-polarized TAMs secrete the chemokine, CCL20 [6], that interacts with its receptor, CCR6, to induce migration and proliferation *in vitro*, and enhance tumor growth and metastasis *in vivo* [7]. Another chemokine secreted from M2-polarized TAMs is CCL2, which also can promote tumor cell migration, invasion, and resistance to therapy by interaction with its receptor, CCR2 [8, 9]. Recent research has focused on strategies to target the immunosuppressive tumor-associated macrophages or the chemokines in order to improve the efficacy of therapeutics for pancreatic cancer [10, 11]. Since the macrophage has an important role in maintaining the health and function of the

normal pancreas, strategies aimed at reprogramming rather than elimination of the TAMs are being explored as promising approaches to disrupt the supportive tumor niche and enhance treatment efficacy in PDAC [11].

We previously showed in a murine model of PDAC that tumors have an abundance of arginase-positive M2-polarized TAMs [12]. In this same animal model, tumors exhibited significantly fewer M2-polarized TAMs in the cohort of mice treated with a cholecystokinin-B receptor (CCK-BR) antagonist proglumide. The reduction in the M2-polarized TAMs was associated with a significant decrease in tumor weight and prolonged survival [12]. This finding prompted us to question how proglumide could change the immune cell signature of pancreatic cancer. In another murine model of chronic pancreatitis induced by a choline-deficient ethionine-supplemented (CDE) high-fat diet, we showed that proglumide decreased tissue inflammation and also reversed biochemical evidence of inflammation with lower pancreatic lipase and amylase serum levels [13]. Mice fed the CDE diet also developed metabolic dysfunction-associated steatohepatitis with histologic and biochemical hepatic inflammation with elevated serum hepatic transaminases [14]. The elevated liver enzymes decreased toward normal when mice were concomitantly treated with proglumide while on the CDE diet, suggesting that proglumide was reversing the inflammation. Since chemokines and cytokines are inflammatory mediators, we examined the expression of these compounds in the tissues of CDE-fed mice with and without proglumide [15]. CCL20 and CCL2 gene expression by PCR increased > 100-fold in tissues of CDE-fed mice compared to standard chow-fed mice, and proglumide therapy significantly lowered both of the chemokine values. In pancreatic cancer and other tissues, M2-polarized macrophages are the primary sources of CCL20 [16]. CCL2 can be released from pancreatic cancer cells and serve as a chemoattractant for macrophages [17], and CCL2 plays an important role in the polarization of TAMs [18].

The purpose of this investigation was to evaluate the mechanisms of cross-communication that influence the polarization of macrophages and how blockade of the CCK-BR with proglumide interrupts these pathways by

inducing plasticity of TAMs and making the pancreatic TME less immunosuppressive.

## Materials and methods

### Ethical regulations

The research conducted in this study complies with all relevant ethical regulations. The animal experiments were carried out in the AAALAC-accredited Comparative Medicine Facility at Georgetown University according to the NIH guidelines for animal care and use and under an approved IACUC protocol.

### Characterization of cell lines

The human pancreatic cancer cell line AsPC-1 (Cat# CRL-1682; RRID: CVCL\_0152), human BxPC-3 pancreatic cancer cells (Cat# CRL-1687; RRID: CVCL\_0186), and human U937 monocytic cells (Cat# CRL-1593.2; RRID: CVCL\_U937) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and obtained from the Georgetown Lombardi Tissue Culture Shared Resource facility. These cells were cultured in humidified air with 5% CO<sub>2</sub> with RPMI-1640 media from Gibco, Life Technologies Corporation, Carlsbad, CA (Cat# 11,875,093); media supplemented with 10% fetal bovine serum (Cat# SH30910.03, CYTIVA, HyClone, Logan, Utah) and 1% penicillin/streptomycin. Human PANC-1 pancreatic cancer cells were purchased from ATCC (Cat# CRL-1469; RRID: CVCL\_0480) and cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Cat# 11,995,065) with supplements as above. Cancer cells were tested and pathogen-free using IMPACT II PCR Profile (IDEXX BioAnalytics Case # 24,470, Columbia, MO).

### *In vitro* polarization of U937 cells

The U937 monocyte cells were differentiated into macrophages (MØ) with 20 ng/ml of phorbol 12-myristate (PMA, ARCOS organics, Cat# 356,150,050) after incubation for 24 h. The MØ phase of the U937 cells was treated with 20 ng/mL IL-10 (ThermoFisher; Cat# AF-200–10-10UG) for 48 h to polarize the MØ phase to the M2 phenotype. MØ polarization was confirmed by measuring RNA with quantitative reverse transcription polymerase chain reaction (qRT-PCR) for human SOCS-1. See PCR primers in Supplementary Table S1. M2-polarization was confirmed by staining cells for arginase-1.

To study the effects of the products from the M2-polarized macrophages on growth of human PDAC cancer cells in culture, we applied two methods. Supernatant from the spent media was collected from chemically polarized the M2-polarized U937 macrophages for application to the pancreatic cancer 3-dimensional spheroids. The second method involved co-culture with cancer cells

and U937 monocytes using the transwell method with inserts placed into the wells.

### Establishment of 3-dimensional cancer spheroids

To recapitulate pancreatic tumors *in vitro*, we developed AsPC-1 and BxPC-3 pancreatic cancer 3-dimensional (3D)-spheroids as previously described [19]. Spheroids were used to study the effects of M2-polarized macrophage supernatant spent media and the effects of co-culture in transwells on PDAC proliferation, differentially expressed genes, and oncogenic signaling pathways. 3D-spheroids were established using the hanging drop protocol established by Foty [20] followed by transferring the spheroids to 12-well low cell attachment plates, (Nunc™ Sphera™ 3D cultureware, Thermo Fisher Scientific; Cat# 174,930) in the presence of a Ready-to-use kit including Basal Medium and SupplementMix, 250 ml (Sigma; Cat# C-28070) and 3D Tumorsphere Medium XF (Promocell; Cat# C28070) containing growth factors and supplements. After the pancreatic cancer 3D-spheroids were established, they were placed in transwell inserts and subjected to co-culture with U937 cells or application of M2-polarized U937 spent media. In the co-culture experiments, AsPC-1 spheroids were placed in the inserts (VWR inserts Cat#734–2731) with U937 cells outside the basket insert in the wells. The wells had a 4 µm size membrane to prevent cells from traversing the barrier and having direct contact. After 72 h after exposure to M2-polarized spent media or co-culture in transwells, the spheroids were aspirated, disrupted, and viable cell counts were done by the trypan blue exclusion technique. In the co-culture wells, the attached U937 cells were collected and stained with arginase-1 (titer 1:100; Invitrogen, Cat# PA5-29,645) and visualized by microscopy to identify M2-polarized macrophages. The adherent and non-adherent U937 cells from additional wells were collected separately and were stained with fluorescent antibodies and then subjected to flow cytometry to determine if the adherent U937 cells were M2-polarized, (using CD206 antibody, an M2 marker) or were unpolarized M0 or M1-polarized (with CD80 and CD86 antibodies) (See Supplementary Table S2, surface antibodies for flow cytometry). Next, the AsPC-1 spheroids were exposed to the CCK-BR antagonist, proglumide (20 nM) and the MTT viability assay was performed after 48 h of exposure. BxPC-3 3D-spheroids were also co-cultured in the transwell plate apparatus alone or in co-culture with U937 cells with and without proglumide. After 72 h, the BxPC-3 cells were counted with trypan blue technique and number of viable cells compared between treatment groups.

### Differentially expressed genes by RNA sequencing

RNA was extracted from untreated AsPC-1 spheroids, from spheroids exposed to the supernatant from the M2-polarized U937 cells spent media, from spheroids exposed to co-culture with U937 cells, or spheroids exposed to proglumide (20 nM) for evaluation of differentially expressed genes (DEGs). The RNA integrity and quantitation were assessed using an RNA Nano 6000 Assay Kit with the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). Only samples with RIN values >8.0 were utilized for RNAseq analysis. Library preparation for transcriptome sequencing was performed by Novogene Co., Ltd. (Sacramento, CA) with 1 µg RNA per sample on a NovaSeq6000 sequencing machine. Further analysis and visualization were performed with Ingenuity Pathway Analysis (Qiagen, Hilden, Germany) and R programming language. Differentially expressed genes were identified with a logFC between -0.5 and +0.5 with an adjusted p-value of less than 0.05, correcting for false discovery via the Benjamini–Hochberg method.

### Evaluation of differentially expressed protein signaling pathways by reverse phase protein array (RPPA)

AsPC-1 3-D spheroids and supernatant exosomes were collected and analyzed by reverse phase protein array (RPPA) to compare signaling profiles in untreated cancer spheroids, spheroids co-cultured with U937 cells, and spheroids after treatment with proglumide (20 nM) × 72 h.

Cells were lysed as previously described and diluted to a final protein concentration of 500 µg/mL [21]. Cell lysates were then immobilized onto a solid substrate, nitrocellulose-coated glass slides (Oncyte Avid, Grace Bio-Labs, Bend, OR, USA), using an Aushon 2470 arrayer (Quanterix, Billerica, MA). Samples were printed in technical replicates along with reference standards used for internal quality control purposes [22]. Selected arrays were used to determine protein concentration in each sample using Sypro Ruby Protein Blot Stain (Molecular Probes, Eugene, OR) [23], and derived protein amounts were used for normalization purposes. Slides were blocked as previously described [23] and immunostained using an automated Epredia Autostainer 360 system (DA Breda, Netherlands). In brief, arrays were blocked with 3% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO), an avidin/biotin blocking solution (Dako Cytomation, Carpinteria, CA), and an additional serum-free protein block (Dako Cytomation, Carpinteria, CA) followed by incubation with previously validated polyclonal and monoclonal antibodies [24] (Supplementary Table S3). Biotinylated secondary antibodies (Vector Laboratories, Inc. Burlingame, CA) followed by tyramide-based amplification coupled with fluorescent detection were used to

quantify signaling molecules within each sample as previously described [21]. Antibody and Sypro Ruby-stained slides were imaged using a Tecan laser scanner (TECAN, Mönnedorf, Switzerland) and analyzed as previously described [25]. RPPA intensity units were represented using unsupervised hierarchical clustering generated with JMP Pro v19 where samples were normalized using the Ward's method.

### Western blot analysis of spheroids in co-culture with and without proglumide

AsPC-1 or BxPC-3 spheroids were placed in the transwell insert with 500ul 3D medium. U937 cells ( $10^6$ ) in 1 ml RPMI complete medium were added to the outer well of the 12-well plate, with or without proglumide (20 nM). After 72 h, the spheroids were harvested from the basket insert and centrifuged (1000 rpm for 5 min). Spheroid lysate was prepared with RIPA buffer (Thermo-Fisher/Pierce, Cat# 89,900) containing protease inhibitor (Pierce, Cat# A32955). The protein concentration was measured using BCA protein assay kit (Pierce, reagent A, Cat# 23,228 and reagent B, Cat# 1,859,078) and using an albumin standard (Thermo Fisher Scientific, Cat# 23,209). Protein (100 ng) in NuPAGE LDS sample buffer (Invitrogen, Cat# NP0007) in a volume 25ul was loaded to the NuPAGE 4–12% Bis–Tris Gel (Invitrogen, Cat# NP0323BOX) and submitted to gel electrophoresis then transferred to a Nitrocellulose membrane (TransBlot Turbo, Cat# L002049B) using a transfer machine (Trans-Blot Turbo Transfer System, BIO-RAD). The membrane was blocked in 5% BSA (albumin, bovine powder, VWR Life Science, Cat# 0332–100 g) in TBST buffer (Intelix-Bio, Cat# DBOC2005BF) for one hour at room temperature, followed by exposure to each primary antibody (1:1000; in 2.5% BSA in TBST buffer) shaking for overnight at 4 °C. After washing the membrane with TBST buffer × 3 for 10 min/ea, then the membrane was incubated with the secondary antibody (Goat Anti-Rabbit IgG Secondary Antibody HRP Conjugated, SAB Signalway Antibody, Cat# L3032, diluted in 2.5% BSA at 1:5000) at room temperature for one hour, then the membrane was washed with TBST buffer 3 × at 10 min/ea. The primary antibodies included Phospho-Akt (Thr308); SOX2; Phospho-PKCδ(T505); (Supplementary Table S4) and the normalizer was GAPDH (Cell Signaling, Cat# 5174).

### Downregulation of *ELANE* expression

Three unique human shRNA (29mer each) plasmid constructs for human *ELANE* (neutrophil elastase) in retroviral untagged vector (5 µg each) were purchased from OriGene Technologies (Rockville, MD; Cat# TR313237) along with a scrambled control. The sequences of the shRNAs are shown in Supplementary Table S5. AsPC-1 cells were transfected with Lipofectamine™-3000 (Cat#

L3000001; ThermoFisher, Rockville, MD) in serum-free Opti-MEM media for 24 h followed by selection media containing puromycin (0.5 µg/ml). RNA was extracted from transfected cells and knockdown was evaluated by qRT-PCR for *ELANE* using selective primers (Supplementary Table S1). Single-cell cloning of the *ELANE*-knockdown cells was performed after repeat shRNA knockdown was done to obtain further knockdown of the mRNA, referred to as knockdown-2. *ELANE* mRNA expression by PCR was measured in AsPC-1 spheroids with and without co-culture with U937 cells. *ELANE* expression was also measured by PCR in U937 cells in suspension and U937 adherent cells after co-culture with AsPC-1 spheroids. Total RNA was extracted using miRNA extraction kit (miRNeasy Mini Kit, QIAGEN, Cat# 1,038,703) and converted to cDNA (qScript cDNA SuperMix, QuantaBio, Cat# 95,048–025). PCR was programmed at 1 cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min on a StepOne Plus Real-Time PCR System, (Applied Biosystems).

#### Co-Culture experiments with *ELANE*-KD AsPC-1 cells and U937 cells

Using the co-culture system with transwell inserts in multi-well plates, we performed a series of experiments to determine if the downregulation of *ELANE* expression could prevent polarization of U937 monocytes to M2-polarized macrophages. U937 monocytes were co-cultured with either AsPC-1 wild-type spheroids or *ELANE*-KD AsPC-1 spheroids placed in the transwell inserts and the U937 cells in the surrounding media. A round glass coverslip was placed at the bottom of each well. After 72 h of co-culture, the coverslips were stained with arginase-1 antibody to determine M2-polarization phenotype (1:100; ThermoFisher, Cat# PA5-29,645) and the number of arginase-positive cells examined by microscopy.

#### Animal experimental models

In vivo studies were performed to compare the growth of pooled *ELANE*-knockdown (KDx1) AsPC-1 cells from shRNA-B and AsPC-1 cells from the *ELANE*-knockdownx2 clonal cells compared to wild-type AsPC-1 control cells. Tumors were formed by injecting cells ( $10^6$ ) in a volume of 100 µl subcutaneously into the right and left flanks of ( $N=10$ ; ~8 weeks of age and 25 g) female athymic nude mice (#553, NCI Ath/nu MOUSE, Charles River Laboratories, Rockville, MD). Tumor volumes were measured weekly with calipers and after 4 weeks the experiment was terminated when control tumors reached the allowed size per the protocol.

#### Immunohistochemical analysis of AsPC-1 tumors for immune cells

Tumor sections (5 µm) from paraffin-embedded AsPC-1 pancreatic tumors were prepared. Immunohistochemical staining for M2-polarized macrophages, M1-polarized macrophages, proliferation index (Ki67), and N1-polarized tumor-associated neutrophils (FasR) was performed. Briefly, slides were treated with 3% hydrogen peroxide and 10% normal goat serum for 10 min each, and exposed to primary antibodies for 1 h at room temperature (see Supplementary Table S6) followed by exposure to horseradish peroxidase-labeled polymer; Dako K4003) according to the manufacturer's instructions. Images were taken on an Olympus BX61 microscope with a DP73 camera. The number of immunoreactive cells per slide area was counted with Image-J computer software by an assistant blinded to the treatments. To distinguish human cancer epithelial cells from the macrophages, wild-type tumor sections were reacted with Akoya labeled antibodies (see Supplementary Table S6).

#### Flow cytometry analysis of AsPC-1 tumors for immune cells

Since N2-polarized neutrophils and M2-polarized macrophages can both stain positively with arginase-1 in tissues by immunohistochemistry, we utilized flow cytometry to selectively gate for M2-polarized macrophages and N2-polarized neutrophils. Tumor sections of the AsPC-1 tumors in each cohort were dissociated and subjected to flow cytometry. Using a CD86 antibody (BioLegend; Cat# 105,061) and an arginase-1 antibody (Invitrogen; Cat# 12–3697-82) we used flow cytometry to gate selectively for M2-polarized TAMs. To identify M1-polarized TAMs we gated with a CD86 antibody and an MHC Class II antibody (BioLegend; Cat# 107,621). To differentiate arginase+ N2-polarized neutrophils from arginase+ M2-polarized macrophages, we selectively gated with a Ly6G antibody (BioLegend; Cat# 127,623) for neutrophils and arginase-1 antibody. The gating strategy for macrophages was as follows: Scatter gate, viability gate, CD45 positive, F4/80 and CD68 double positive to identify macrophages. Cell were then evaluated with specific M1/M2 markers. The gating strategy for the neutrophils was as follows: Scatter gate, viability gate, CD45 positive, Ly6G positive to identify neutrophils; then evaluated for arginase. Cell viability was confirmed with Zombie Staining (BioLegend, Cat# 423,109). The panel of flow cytometry antibodies is provided in the Supplementary data Table S7.

#### Analysis of cancer cell surface receptors by flow cytometry

AsPC-1 cells were analyzed for the following cell surface G protein coupled receptors (GPCRs) by flow cytometry: CCK-BR, CCR6, and CCR2. PANC-1 cells were also analyzed for surface receptors by flow cytometry with the

CCK-BR and CCR2 antibodies. The information on the anti-human flow cytometry antibodies is found in Supplementary Table S2.

#### GPCR crosstalk experiments

AsPC-1 cells were cultured in 6 well plates and treated with CCL20 (10ug/ml; Sigma, Cat# SRP4491-20UG) or CCL2 (15 ng/ml) (also called MCP1; Invitrogen, Cat# RP-8648) in the presence or absence of the CCK receptor antagonist proglumide 20 nM (Tocris Biosciences; Cat#1478/50). There were  $N=6$  wells/treatment. Control cells were untreated. Media was collected 24 h after exposure to the chemokines CCL20 and CCL2 and assayed by enzyme-linked immunosorbent assay (ELISA) for secretion of TNF $\alpha$  (Human TNF-alpha SimpleStep ELISA® Kit Abcam, Cat# ab181421) or interferon- $\gamma$  (Human Interferon-gamma SimpleStep ELISA® Kit Abcam, Cat# ab300323), respectively. Samples were assayed in triplicate and read at an OD of 450 nm on a plate reader.

#### Statistical analysis

Sample size for wild-type and knockdown *in vivo* studies includes  $N=10$  tumors per group based upon our prior studies in WT tumors. For the RPPA data, each sample was printed in technical replicates ( $N=3$ ) along with reference standards for QC/QA purposes consisting of a 6–10-point curve whereby the analyte of interest is decreasing in concentration in the background of a constant protein concentration.

Graphical design was done using GraphPad Prism version 10.0 with graphical analysis using Student's *t*-test when comparing two groups with normal distribution. One-way ANOVA was used for group comparisons, and a Bonferroni adjustment was made for all parametric analyses that had more than one comparative group compared to the control.

The human RNA sequencing data were analyzed with Qiagen Ingenuity Pathway Analysis (IPA) for bioinformatics analysis, which included canonical pathway analysis, disease and function, regulator effects, upstream regulators, and molecular networks. IPA uses a network generation algorithm to segment the network map between molecules into multiple networks and assign scores for each network. The network overlap function was used to generate a network that connects pathways that had overlapping genes.

Criteria used to analyze the common pathways between the three spheroid treatment groups and controls included the following for IPA analysis of all common pathways: based upon the following criteria for gene expression: the standard: proglumide group ( $P<0.05$ ,  $\log_2 \text{FC} \leq -1$  or  $\geq 1$ , protein coding), U937 co-culture genes ( $P<0.05$ ,  $\log_2 \text{FC} \leq -1.5$  or  $\geq 1.5$ , protein coding) and spent medium genes ( $P<0.05$ ,  $\log_2 \text{FC} \leq -2$  or  $\geq 2$ ,

protein coding). These same criteria were used when we analyzed the specific common pathways and genes involved in apoptosis and the tumor microenvironment of the three RNAseq data cohorts by IPA.

Real-time PCR results were analyzed using a Student's *t*-test on the normalized mean  $\Delta\text{CT}$  (the difference between the cycle counts of the gene of interest minus the count of an endogenous control) values for each group, with Bonferroni corrections applied to adjust for multiple comparisons. Analysis of the immunohistochemistry data was done using the Welch Two Sample *t*-test to show significant differences between the groups.

To determine whether we could use the mean cell count of each tumor to evaluate histologic cohort differences between wild-type and *ELANE*-KD tumors, we first assessed the within-tumor variance using Levene's test to assess the homogeneity of variance within each group. To evaluate whether there was a statistically significant difference in the number of Arginase+ cells between the wild-type and *ELANE*-KD cohorts, normality of the data was assessed using the Shapiro–Wilk test, and the data results indicated that neither group violated the assumption of normality. Therefore, both unpaired two-tailed Student's *t*-test and Wilcoxon rank-sum test were performed to compare the groups.

## Results

### Macrophage co-culture and supernatant from spent media from M2-polarized macrophages increase the proliferation of 3-dimensional pancreatic cancer spheroids

We used two methods to analyze the influence of M2-polarized macrophage-derived products on pancreatic cancer cells *in vitro*: co-culture and the application of the supernatant from the chemically-polarized M2 macrophage spent media. Using standard protocols to polarize monocytes into M $\phi$  macrophages or M2 macrophages *in vitro* [26, 27], human U937 monocyte cells were polarized to M2-polarized macrophages (Fig. 1A). After the treatment with phorbol myristate acetate (PMA), polarization from M0 to M $\phi$  macrophages was confirmed by qRT-PCR for SOCS-1 expression (Fig. 1B, top), and after IL-10 treatment we confirmed M2-polarization of the U937 cells by arginase-1 staining (Fig. 1B, bottom).

Many types of *in vitro* three-dimensional (3D) culture systems have been developed to recapitulate the *in vivo* growth conditions of cancer [28] including tumorspheres (spheroids) and organoids. Shi et al. [29], previously demonstrated that human AsPC-1 pancreatic cancer cells readily form 3-D spheroids that are 360 to 500  $\mu\text{m}$  in diameter and represent an ideal model for studying signaling pathways and therapeutics in the laboratory. Using these *in vitro* protocols, we developed 3D-spheroids from human AsPC-1 cancer cells and the established spheroids

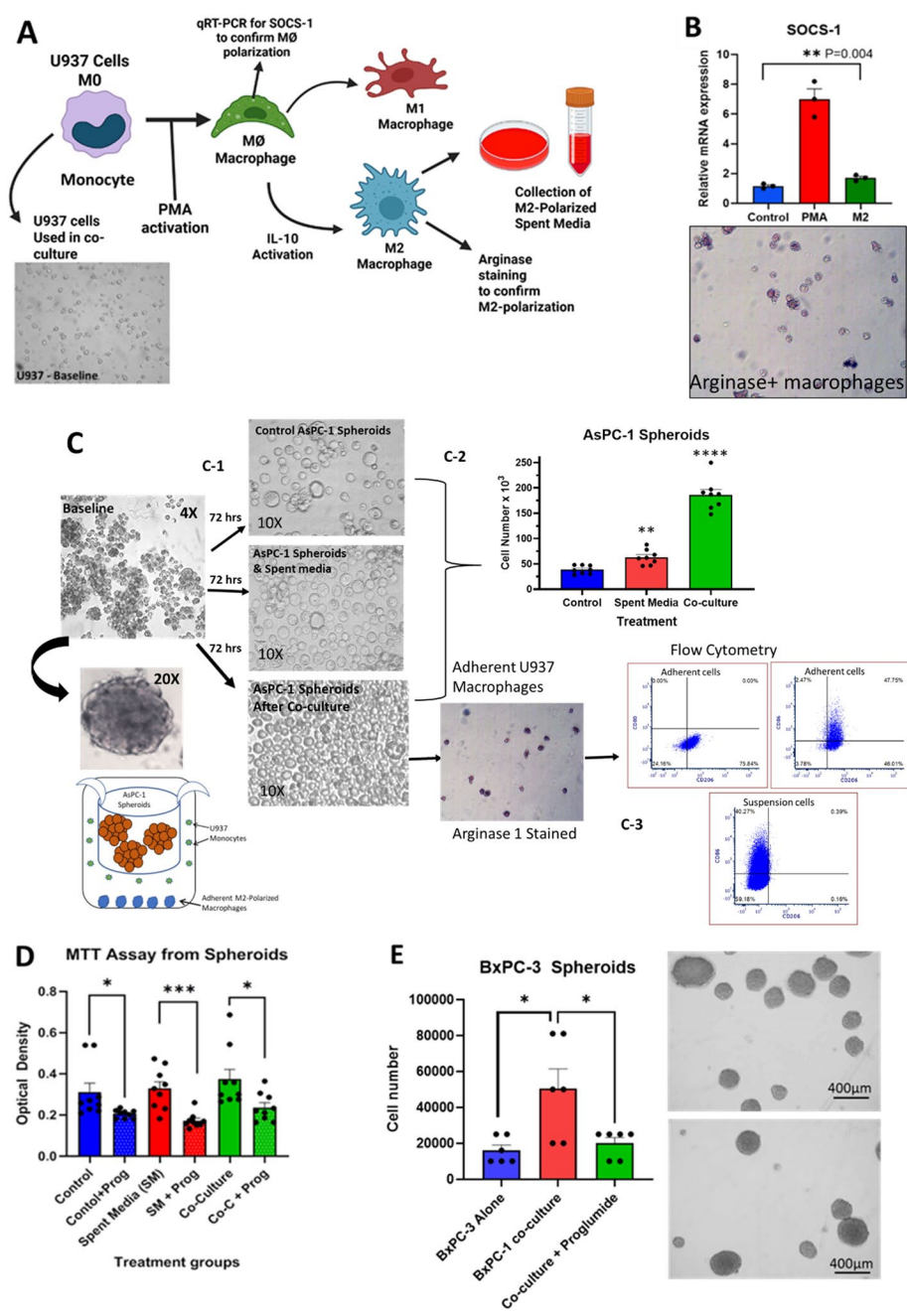


**Fig. 1** (See legend on next page.)

were grown in the transwell inserts with 4µm pore size base in the 12-well plate (diagram at bottom of Fig. 1C). Supernatant from the spent media was collected from the chemically-induced M2-polarized macrophages for use in the *in vitro* growth assays. The effect of M2-polarized spent media on human PDAC spheroids was analyzed and compared to that of fresh media controls (Fig. 1C). Additionally, the effect of co-culture of U937 monocytes (not chemically treated) with PDAC spheroids was also evaluated. When AsPC-1 spheroids were treated with supernatant spent media from M2-polarized U937 cells

or were co-cultured in the transwells with the U937 cells in the surrounding media, the spheroids proliferated. Representative photos of the spheroids for each condition are shown in Fig. 1C-1. The number of total cells in the spheroids treated with the supernatant from the M2-polaized spent media increased by approximately twofold over untreated controls (Fig. 1C-2, red column). These results suggest that a component of the supernatant spent media from the *in vitro* chemically derived M2-polarized U937 cells communicated with the cancer spheroids to induce cell growth. When the AsPC-1

(See figure on previous page.)

**Fig. 1** Proliferation of pancreatic cancer spheroids and polarization of U937 cells in transwell culture. **A** U937 monocyte cells were treated with PMA to chemically polarize to M0 macrophages. Then M0 cells were chemically polarized with IL-10 to M2 macrophages. Spent media supernatant was collected from M2 macrophages. Cells were stained with arginase to confirm M2-polarization. Figure created in BioRender, Smith, J. (2025); <https://BioRender.com/fquwsxl>. **B** Top figure: RNA was collected from the M0 macrophages to confirm SOCS-1 expression by qRT-PCR. PCR was done in replicates of three. Analysis by one-way ANOVA; Significance of  $**P=0.004$ . Bottom figure: Confirmation of M2-polarization of U937 cells after IL-10 was confirmed by arginase staining. **C** Effects of co-culture or spent media supernatant on growth of AsPC-1 pancreatic cancer spheroids. **C-1** Appearance of AsPC-1 spheroids shown at baseline at low magnification (4X) and at a higher magnification (20X). A cartoon of the transwell co-culture is shown below, bottom left. Representative photos show AsPC-1 spheroids after 72 h when grown alone (control-top), grown in culture with spent media supernatant from M2-polarized U937 cells (middle), or when grown in co-culture in transwell plates with U937 cells (bottom). After 72 h the spheroids were aspirated, dissociated and cell numbers counted in each group (replicates of three). **C-2** The graphical results show columns representing mean  $\pm$  SEM for each group with increased AsPC-1 cell number in these spheroids exposed to spent media supernatant or grown in co-culture compared to controls. Analysis was done by Student's t-test with Bonferroni correction for multiple comparisons to controls.  $**P<0.01$  and  $****P<0.0001$ . **C-3** The U937 cells became adherent in the co-culture dish and were stained with arginase to confirm M2-polarization, left. The adherent macrophages were also collected and stained with the M2 macrophage antibody CD206 and with the M0/M1-macrophage antibodies CD80 and CD86 and subjected to flow cytometry, right. The non-adherent cells from the suspension were stained with CD206 and CD86 and subjected to flow cytometry. **D** Effects of proglumide treatment on MTT viability assay of AsPC-1 cells from spheroids that were untreated (Controls), treated with spent media (SM) or were co-cultured (Co-C) with U937 cells. Columns represent means  $\pm$  SEM and in replicates of nine. Analysis of each treatment groups was done by student's two-way t-test. Significance =  $*P<0.05$ ;  $***P<0.005$ . **E** Growth of human pancreatic cancer BxPC-3 spheroids in transwell inserts alone or in transwell inserts grown with U937 monocytes in co-culture with or without proglumide treatment. Columns represent mean values from  $N=6$  wells each. Significant differences were analyzed by student's two-way t-test;  $*P<0.05$ . Representative images of BxPC-3 spheroids are shown to the right

spheroids were co-cultured with untreated U937 monocytes that had not been chemically treated to induce polarization, the number of AsPC-1 spheroids increased fivefold compared to controls (Fig. 1C-2, green column). When co-cultured with the pancreatic cancer spheroids in the transwell apparatus, the U937 monocytes polarized to M2-macrophages and the polarization was confirmed by their adherence to the bottom of the culture plate (rather than remaining in suspension) and also by positive arginase-1 staining (Fig. 1C-3). The adherent U937 cells and the non-adherent U937 cells from the co-culture experiments were collected separately and subjected to flow cytometry with macrophage/monocyte antibodies to confirm that the arginase-positive adherent cells were indeed M2-polarized macrophages. Figure 1C-3, right shows results of the flow cytometry of the adherent cells confirming that 93.76% are M2-polarized and stain with CD206. None of the adherent cells stained with the CD80 antibody, an M0 marker and only 2.46% of the adherent cells stained solely for CD86, an M0 and M1 marker. The U937 cells that were still in suspension (i.e., non-adherent) and stained with the antibodies showed that less than 1% reacted with the CD206 (the M2 marker) but >99% stained with CD86 which reacts with unpolarized (M0) macrophages and M1 macrophages (Fig. 1C-3). The marked increased proliferation of the cancer spheroids when co-cultured with the U937 cells suggests that the monocytes directly influenced the growth of malignant cells perhaps by releasing growth factors in the media. Likewise, the cancer spheroids most likely released soluble factors into the media to make the U937 monocytes polarize to the M2-phenotype allowing proliferation of the cancer cells.

To evaluate whether CCK-BR blockade could interrupt the proliferative effects of AsPC-1 spheroids from M2-polarized macrophage, each treatment group was

next exposed to media with and without proglumide for 48 h. Proglumide treatment decreased the viability of the AsPC-1 cell in all three conditions: control spheroids, spent-media-treated spheroids, and co-cultured spheroids (Fig. 1D) as measured by the MTT assay. In the control spheroids, proglumide most likely decreased proliferation by blocking CCK-BR and its downstream ERK, MAPK, and RAS signaling pathways (to be examined below). In the cells treated with proglumide and supernatant of M2-spent media, the inhibitory effects of proglumide were more pronounced perhaps since proglumide not only blocked the downstream proliferative signaling pathways, but it also interrupted the effects of chemokines, like CCL2 and CCL20, in the spent media supernatant. In the co-culture experiment, proglumide most likely interrupted the communication by exosomes or other growth factors released from the cancer cells. This finding is discussed below in regard to the detection of neutrophil elastase in the AsPC-1 secreted exosomes.

To validate that pancreatic cancer spheroids, proliferate in co-culture with U937 cells, spheroids were also established from human BxPC-3 pancreatic cancer cells. When BxPC-3 spheroids in the transwell inserts were exposed to U937 cells in the surrounding media, the BxPC-3 spheroids also increased in number (Fig. 1E) and this effect was blocked by proglumide. Representative images of the BxPC-3 spheroids are shown (Fig. 1E).

### Differentially expressed genes by RNA sequencing from 3-D pancreatic cancer spheroids

Total RNA was extracted for RNA sequencing to analyze differentially expressed genes (DEGs) from the AsPC-1 spheroids from the three treatment groups: 1) treatment with proglumide, 2) exposure to supernatant spent media from M2-polarized macrophages, or 3) co-culture with U937 cells, as compared to untreated 3D AsPC-1

spheroid controls. Volcano plots of the DEGs from AsPC-1 3-D spheroids treated with proglumide compared to controls are shown in Fig. 2A with 182 genes upregulated and 724 genes downregulated by proglumide. Since more genes are downregulated, this reflects the inhibitory effects of proglumide by antagonizing the CCK-BR. The volcano plot for the DEGs in the spheroids treated with supernatant spent media from M2-polarized macrophages is shown in Fig. 2B with similar numbers of up- and down-regulated genes. The volcano plot for the DEGs for the AsPC-1 spheroids co-cultured with U937 cells is shown in Fig. 2C with 2,045 upregulated genes and only 298 downregulated genes suggesting the activation of many genes with the co-culture environment. *ELANE* (arrow, 2C), a gene that codes for neutrophil elastase, was the most upregulated gene +17.5 Log2fold change (p-adjusted value  $1.82 \times 10^{-66}$ ) in AsPC-1 spheroids with co-culture with U937 cells.

Heat maps for each group with the top 20 up- or down-regulated protein-coding genes in AsPC-1 spheroids are shown in Figs. 2D-F for proglumide treatment, supernatant spent media, and co-culture, respectively. The two most downregulated genes by proglumide that were significantly downregulated by  $-7$  Log2fold include *NPIP88* (Nuclear pore complex interacting protein family member B8, also known as NUP88) and *PLA2G4F* (Fig. 2D). NUP88 has been shown to affect gene expression in cancer cells by influencing the localization and activity of transcription factors, playing a role in the transport of proteins and mRNA between the nucleus and cytoplasm, a process that is often dysregulated in cancer [30]. *PLA2G4F* is specifically expressed in malignant cells and functions as a cell-autonomous metabolic reprogramming factor. *PLA2G4F* has been shown to promote the proliferation, migration, and survival of triple-negative breast cancer *in vitro* [31]. Another gene significantly decreased by proglumide treatment ( $-6$  Log2fold decrease) is *KIF1a*, a gene that is a member of the kinesin superfamily of proteins associated with a poor prognosis in cancer [32]. *COL4A3* is downregulated ( $-6.1$  Log2fold) in spheroids with proglumide therapy. Collagen IV is regulated by the *COL4A3* gene and is an important part of the extracellular matrix basement membrane; increased expression of *COL4A3* in pancreatic cancer is associated with the propensity of the tumor to metastasize to the liver [33, 34]. In contrast, the *CD83* gene is upregulated (+5.9 Log2fold) in spheroids treated with proglumide. The *CD83* molecule plays a very important role in controlling and restoring immune responses and the subsequent induction of the resolution of inflammation. Increased tumor cell expression of *CD83* contributes to suppression of tumor growth and is associated with enhanced cytotoxic T-cell profiles and activated CD8<sup>+</sup> T cells [35]. The *JSRP1* gene codes for junctional

sarcoplasmic reticulum protein and was increased (+6.3 Log2fold) in AsPC-1 spheroids after proglumide treatment. *JSRP1* is a telomere-related gene [36] that has been associated with a favorable outcome and survival in cancer.

In the AsPC-1 spheroids treated with M2-polarized macrophage supernatant spent media *MMP3* was the most upregulated gene by RNAseq, by +11 Log2fold (Fig. 2E). *MMP3* codes for matrix metalloproteinase 3 protein and is strongly associated with oncogenic *KRAS* in orchestrating pancreatic cancer tumorigenesis while priming the activated stroma and serving as a critical driver of tumor invasion and progression [37]. This increased expression supports that the media from M2-polarized TAMs is helping to promote pancreatic tumor growth and affecting the stroma. In AsPC-1 spheroids co-cultured with U937 cells the most upregulated gene, *ELANE* (as mentioned above), is outlined (Fig. 2F). Another gene that codes for the transcription factor CCAAT/enhancer-binding protein alpha (C/EBP $\alpha$ ) that is responsible for activation of the *ELANE* promoter was increased over fourfold with co-culture with an adjusted p-value of  $2 \times 10^{-15}$ . The mRNA expression of C/EBP $\alpha$  was reversed when the spheroids were treated with proglumide.

#### Analysis of protein signaling pathways by reverse phase protein array (RPPA)

RPPA was then utilized to study the protein signaling pathways altered in AsPC-1 spheroids when co-cultured with U937 cells or treated with proglumide. A complete unsupervised hierarchical clustering capturing the proteins changed when AsPC-1 spheroids were co-cultured with U937 cells or when treated with proglumide compared to untreated control spheroids is shown (Supplementary data Fig. S1). The raw data for each sample are provided in Supplementary Table S8. Many of these protein pathways activated by the co-culture with U937 cells were reversed toward the control levels when the spheroids were treated with proglumide.

A magnified section of the RPPA proteins highlighting significant changes with proglumide is shown in Fig. 2G. Untreated AsPC-1 spheroids showed decreased phosphorylation of the CCK-BR downstream signaling substrates PKC delta (T505), pan PKC beta-II (S660), AKT and p21-activated kinase 1 and 2 (PAK-1, PAK-2) [38] in response to proglumide treatment compared to cancer cells co-cultured with U937 cells. A decreased expression was also identified in inflammatory signaling molecules (STAT2 and IL-10), stem cell markers (SOX2), and receptor tyrosine kinases, including EGFR, that we previously identified as a signaling molecule potentially involved in a crosstalk with CCK-BR [39]. Three selected pathways (AKT, PKC $\delta$ , and SOX2), outlined by the

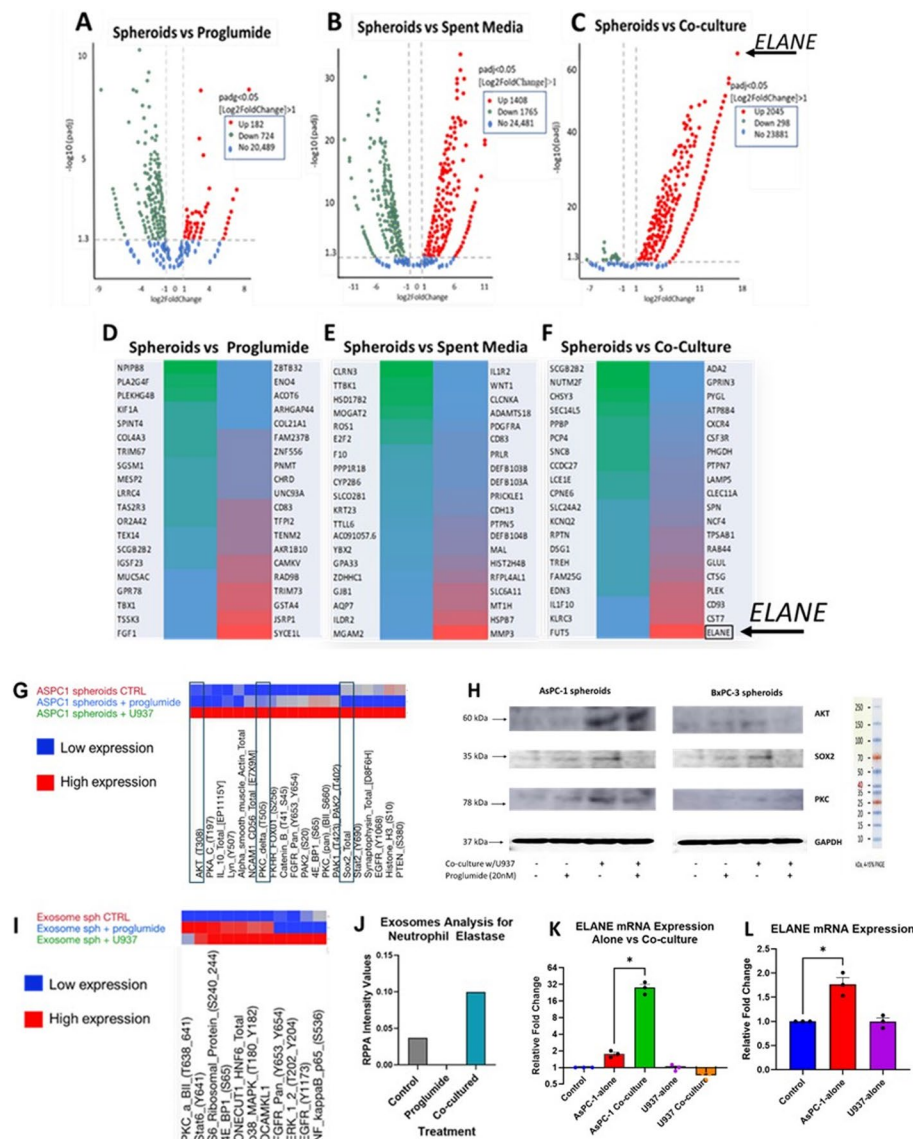


**Fig. 2** Differentially expressed genes and proteins by RNA sequencing and RPPA in AsPC-1 spheroids. In the top row volcano plots of differentially expressed genes for each treatment group is shown. **A** Genes ( $N=724$ ) were significantly downregulated and 182 genes were upregulated in AsPC-1 spheroids treated with proglumide compared to untreated controls. **B** An equal number of downregulated and upregulated genes were seen in AsPC-1 spheroids treated with supernatant spent media compared to controls. **C** There were 2045 upregulated genes and only 298 downregulated genes seen in AsPC-1 spheroids treated in co-culture with U937 cells. The most upregulated gene was *ELANE* (arrow). **D** Heat map for the top 20 up- (in red) and top 20 down- (in green) regulated genes comparing control spheroids to spheroids treated with M2-polarized macrophage supernatant spent media. **E** Heat map for the top 20 up- and top 20 down-regulated genes comparing control spheroids to spheroids treated with M2-polarized macrophage supernatant spent media. **F** Heat map for the top 20 up- and top 20 downregulated genes comparing control spheroids to spheroids co-cultured with U937 cells. **G** Reverse phase protein array (RPPA) of AsPC-1 spheroids shows unsupervised hierarchical clustering capturing the proteins changed when AsPC-1 spheroids were co-cultured with U937 cells or when treated with proglumide compared to untreated control spheroids. The rectangular boxes are highlighting some key proteins and signaling pathways involved in pancreatic cancer cell proliferation that are increased with co-culture with macrophages and decreased with proglumide. **H** Western blot is shown of the three outlined proteins from panel G. The western blot was performed with AsPC-1 and BxPC-3 spheroids that were grown alone or in co-culture with U937 cells with and without proglumide. **I** Unsupervised hierarchical clustering capturing the proteins changed in the isolated exosomes when AsPC-1 spheroids were co-cultured with U937 cells or when treated with proglumide compared to untreated control spheroids. **J** RPPA analysis of pooled exosomes from triplicate replicates of untreated spheroids, co-cultured spheroids, or spheroids treated with proglumide for neutrophil elastase. **K** Relative mRNA expression of *ELANE* by qRT-PCR in AsPC-1 spheroids and U937 cells grown alone or in co-culture.  $*P < 0.05$ . **L** Shows relative mRNA expression of *ELANE* without co-culture in AsPC-1 or U937 cells.  $*P < 0.05$

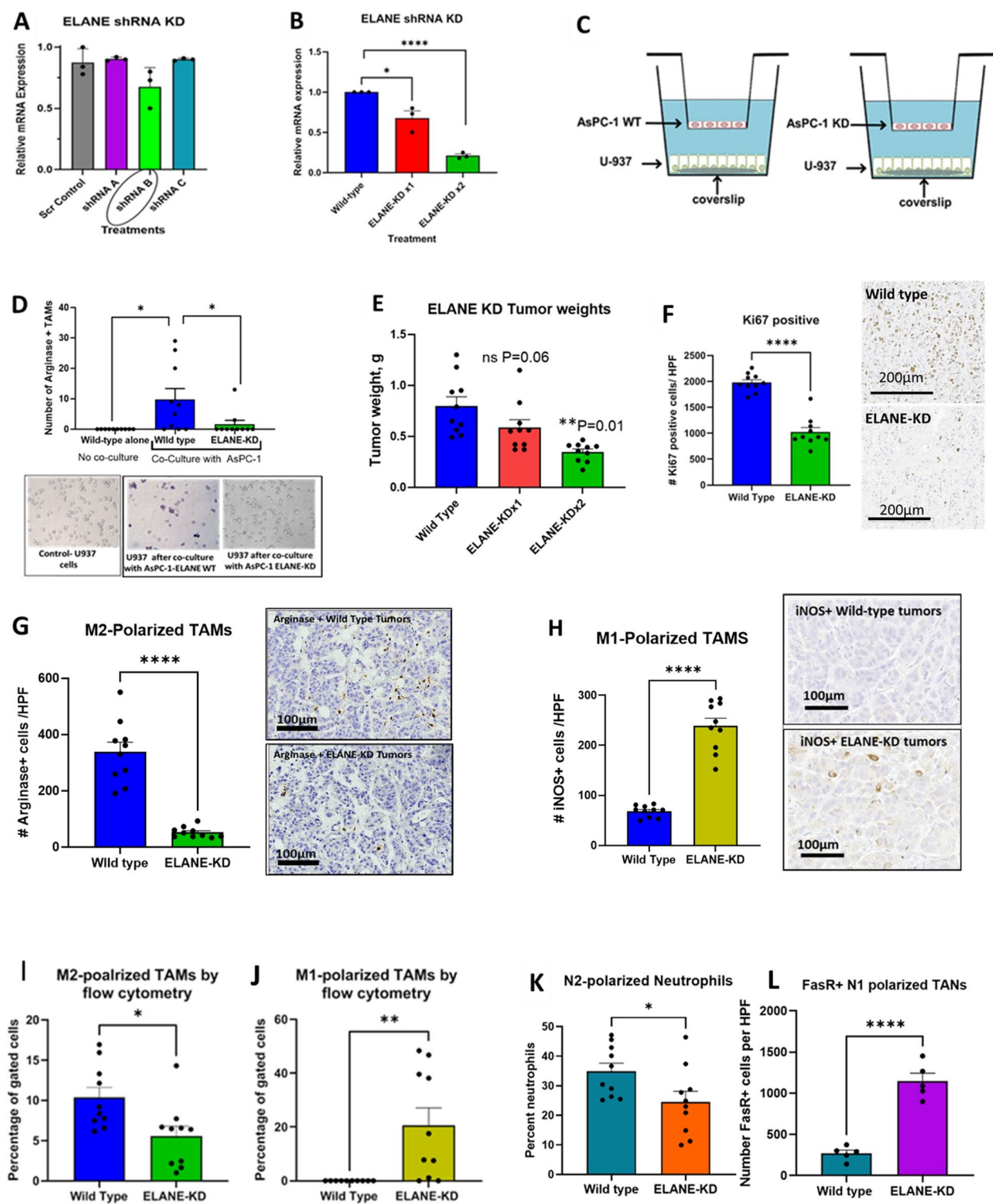


**Fig. 3** (See legend on next page.)

rectangles in Fig. 2G, were further evaluated with western blotting in AsPC-1 and BxPC-3 spheroids (Fig. 2H). The western blot confirms that the expression of these three proteins increase in co-culture with U937 cells and decrease with proglumide.

(See figure on previous page.)

**Fig. 3** Effects of downregulation of *ELANE* mRNA expression on tumor growth and on the tumor microenvironment. **A** Relative mRNA expression of *ELANE* was measured by qRT-PCR after transfection with each of three shRNAs and a scrambled control. shRNA B decreased *ELANE* expression by 32%. **B** qRT-PCR analysis comparing *ELANE* mRNA relative expression in wild-type AsPC-1 cells compared to the first transfection (KD × 1) and the 2nd transfection KD × 2 cells. Assays were done in triplicate and the results were analyzed using a student's t-test on the normalized mean  $\Delta$ CT (the difference between the cycle counts of the gene of interest minus the count of an endogenous control) values for each group, with Bonferroni corrections applied to adjust for multiple comparisons; (\* $P$  < 0.05 and \*\*\*\* $P$  < 0.0001). **C** Schematic diagram showing the study design of the co-culture experiment with wild-type AsPC-1 cells or *ELANE*-KD AsPC-1 cells co-cultured with U937 monocytes ( $N$  = 6 wells per treatment). **D** 72 h after co-culture, the coverslips from the bottom of the tissue culture wells were stained for M2-polarized TAMs with arginase. Bar graph representing the mean  $\pm$  SEM number of the number of arginase positive macrophages adherent on the coverslips per high powered field. None of the U937 monocytes (control) were adherent to the coverslips. Representative images are shown for each group. Assay was done in triplicate. **E** Final tumor weights comparing wild-type AsPC-1 tumors to that of tumors from AsPC-1 *ELANE*-KD × 1 and *ELANE* KD × 2 cells; ( $N$  = 10/group). Columns represent the mean  $\pm$  SEM of the tumor weights in vivo showing the dose response with smaller tumors with the *ELANE* KD × 2 AsPC-1 cells. Significant by student's t-test for each group, with Bonferroni corrections applied to adjust for multiple comparisons (\*\* $P$  < 0.01). **F** Ki67 immunohistochemistry staining for proliferating cells compares wild-type tumors to *ELANE*-KD tumors (\*\*\*\* $P$  < 0.0001). **G** A representative photo (top) from a wild-type AsPC-1 tumor showing numerous arginase + M2-polarized macrophages by immunohistochemistry. The bottom image is a representative picture showing the decreased number of arginase + M2-polarized TAMs in the tumors from the *ELANE*-KD cells. Analysis using Welch Two Sample t-test showed significant differences between the groups (\*\*\*\* $P$  < 0.0001;  $N$  = 10). **H** Representative photos of iNOS stained wild-type tumors (top) and *ELANE*-KD tumors (bottom) for pro-inflammatory M1-polarized TAMs. Analysis using Welch Two Sample t-test showed significant differences between the groups (\*\*\*\* $P$  < 0.0001;  $N$  = 10). **I** Flow cytometry results from samples of sections of wild-type or *ELANE*-KD tumors gated for M2-polarized TAMs (\* $P$  < 0.05) or **J** M1-polarized TAMs (\*\* $P$  < 0.01). **K** LY6G and arginase positive gating was used to detect N2-polarized neutrophils by flow cytometry in wild-type tumors or *ELANE*-KD tumors. Analysis using Welch Two Sample t-test showed significant differences between the groups (\* $P$  < 0.05). **L** N1-positive TANs reacted with FasR show an increase in the number of N1 + —neutrophils in the *ELANE*-KD tumors. (\*\*\*\* $P$  < 0.0001 by Welch Two Sample t-test

#### Analysis of exosomes from cancer cells by reverse phase protein array (RPPA)

RPPA was also performed on exosomes isolated from untreated AsPC-1 spheroids, from exosomes after co-culture with U937 cells, and from AsPC-1 spheroids treated with proglumide. The exosome heat map is shown in Fig. 2I with important pathways identified by RPPA in the exosomes related to cross-communication. Exosomes from co-cultured spheroids exhibited high levels of ERK activity, and these levels were reversed in exosomes isolated from proglumide-treated spheroids. NF- $\kappa$ B phosphorylation was increased in the exosomes, and these levels were reduced to greater than the control levels in exosomes collected from spheroids treated with proglumide. Activation of NF- $\kappa$ B is essential for the initiation and progression of pancreatic cancer [40]. The activation of the canonical NF- $\kappa$ B signaling pathway leads to an influx of inflammatory cells and macrophages, controlled via mediators such as cytokines and chemokines [41]. EGFR protein expression was increased in both the spheroids and exosomes in co-culture and reversed with proglumide.

#### Pancreatic cancer cells communicate with macrophages through neutrophil elastase-rich exosomes

Several mechanisms are utilized by cancer cells to influence other cells in the tumor microenvironment. We also collected exosomes from untreated AsPC-1 spheroids, from spheroids grown in co-culture with the U937 cells, and from spheroids treated with proglumide to analyze for neutrophil elastase. Neutrophil elastase was detected by immunoreactivity from pooled exosomes (replicates of 3) released by untreated AsPC-1 spheroids (Fig. 3J). The RPPA intensity value of neutrophil-rich exosomes

more than doubled after co-culture with U937 monocytes, and strikingly, no immunoreactivity was detected for neutrophil elastase in the exosomes collected from proglumide-treated AsPC-1 spheroids (Fig. 2J). To confirm that the source of the neutrophil elastase was from the AsPC-1 cells and not the U937 cells, we measured the gene expression of *ELANE* independently in each cell line by qRT-PCR and after co-culture in the transwell plates. Similar to the RNA sequencing data, *ELANE* expression was low in AsPC-1 spheroids grown alone but increased about 30-fold after exposed to the U937 cells (Fig. 2K). *ELANE* expression was also low in U937 cells, and basal levels were lower than expression in the cancer cells. However, the expression of *ELANE* did not significantly change after co-culture with the AsPC-1 cancer cells (Fig. 2L). These results imply that the neutrophil elastase detected in the exosomes were most likely derived from the AsPC-1 spheroids with high *ELANE* expression and not the U937 cells.

#### Down-regulation of *ELANE* expression in AsPC-1 cells

Since *ELANE* was the most upregulated gene (+17.5 Log2fold) identified by RNAseq when AsPC-1 spheroids were co-cultured with U937 cells, and the protein that *ELANE* codes for, neutrophil elastase, was increased in exosomes collected from AsPC-1 spheroids after co-culture with U937 cells, we speculated that *ELANE* must be involved in the cross-communication between the macrophages and cancer cells. To determine the role of *ELANE* expression in pancreatic cancer, we chose to decrease the expression of this gene and evaluate the effects on polarization of TAMs and on pancreatic tumor growth. Using selective shRNA vectors to knock-down *ELANE* mRNA in human AsPC-1 cells, we found

that shRNA-B decreased *ELANE* mRNA expression by 32% compared to the scrambled control (referred to as KD×1) (Fig. 3A). Transfection with the other shRNAs -A and -C did not significantly decrease *ELANE* expression. To further decrease *ELANE* mRNA expression, we used a clonal population from the shRNA-B knockdown cells and repeated the transfection, herein referred to as *ELANE*-KDx2 or simply *ELANE*-KD. After the 2nd shRNA treatment, *ELANE* mRNA was decreased by 80% compared to wild-type AsPC-1 cells and this difference was significant ( $P < 0.0001$ ) (Fig. 3B).

#### **Co-culture of AsPC-1 *ELANE*-KD cells prevents M2-polarization of U937 cells in vitro**

Using a transwell co-culture system (Fig. 3C), we compared the effects of *ELANE*-KD AsPC-1 spheroids to wild-type AsPC-1 spheroids on the ability of U937 cells to become adherent and polarize to the M2-phenotype. After 72 h of co-culture with wild-type AsPC-1 cells, the U937 cells became adherent to the coverslips at the bottom of the wells and stained positive with arginase-1. The U937 cells that were co-cultured with the *ELANE*-KD AsPC-1 spheroids did not exhibit arginase 1 immunoreactivity or adhere to the tissue culture plate. The number of arginase-positive cells M2-polarized U937 cells on the coverslips after co-culturing with wild-type AsPC-1 cells was significantly greater compared to the U937 cells co-cultured with *ELANE*-KD AsPC-1 cells (Fig. 3D). None of the control U937 cells grown in wells without co-cultured cells were adherent, but remained in suspension. Representative photos from untreated U937 cells and coverslips reacted with the arginase-1 antibody containing the U937 cells co-cultured with wild-type or *ELANE*-KD cells are shown below each treatment group (Fig. 3D). These results suggest that *ELANE* expression in the cancer cells regulates the polarization of macrophages.

#### **Knockdown of *ELANE* in AsPC-1 cancer cells decreases tumor mass and alters immune cell signature in the tumor's microenvironment in vivo**

In vivo experiments were performed to examine the role of *ELANE*-KD on tumor growth and macrophage polarity in the TME. Tumors were formed subcutaneously in three cohorts of immune-deficient mice using wild-type human AsPC-1 cells, AsPC-1 cells with *ELANE*-KDx1, and AsPC-1 cells with double knockdown, i.e., *ELANE*-KDx2. Tumor volumes were measured weekly, and after four weeks, when the mice bearing wild-type AsPC-1 tumors had tumors that reached 20mm<sup>3</sup>, the experiment was terminated. Tumors from the *ELANE*-KD cells were smaller than those tumors from the wild-type cells in a dose-related fashion with the most reduction in tumor mass found in the tumors with the greatest down-regulation of *ELANE* mRNA (Fig. 3E). The change in tumor

mass correlated with the proliferative index histologically with the wild-type tumors exhibiting a higher proliferation index as measured by the number of Ki67-positive immunoreactive cells per high powered field (Fig. 3F). Fewer Ki67-positive cells were detected in the tumors with *ELANE*-KD.

We investigated the changes in macrophage polarization in the TME in wild-type AsPC-1 tumors compared to *ELANE*-KD tumors. Pancreatic tumors with *ELANE*-KD had significantly fewer M2-polarized TAMs compared to wild-type tumors. The number of TAMs per high-powered field was counted for each tumor and plotted (Graph, Fig. 3G). A representative image from a wild-type tumor shows an abundant number of arginase-positive M2-polarized TAMs (Fig. 3G, top), and a representative *ELANE*-KD tumor section is shown (Fig. 3G, bottom) with significantly fewer M2-polarized arginase-1 positive TAMs. Since M2-polarized TAMs promote pancreatic cancer growth, decreasing the number of M2-polarized macrophages may contribute to the smaller tumors observed in the mice with *ELANE*-KD.

Next, to determine if *ELANE*-KD induces a shift or reprogramming to the macrophages from the pro-cancer to the anti-cancer/pro-inflammatory phenotype, we analyzed the same tumors for M1-polarized TAMs with immunohistochemical staining with an iNOS antibody. The mean number of M1-polarized TAMs was significantly increased in the tumors of the mice with *ELANE*-KD compared to wild-type controls ( $P < 0.0001$ ) with representative images and analysis shown in (Fig. 3H). These results suggest that altering the mRNA expression of *ELANE* in pancreatic cancer cells influences plasticity in TAMs changing their phenotype from pro-cancer (M2) to anti-cancer (M1) and resulting in a reduction of tumor mass.

#### **Assessment of macrophage and neutrophil polarization by flow cytometry**

Since both M2-polarized macrophages and N2-polarized neutrophils can both stain positively with arginase-1, we used flow cytometry to confirm that the arginase-1 positive staining seen by immunohistochemistry in the tumors represented an increase in M2-positive TAMs. With selective cell staining for macrophages with CD86 and arginase-1, flow cytometry gating confirmed that M2-polarized TAMs were significantly decreased in *ELANE*-KD tumors compared to wild-type pancreatic tumors (Fig. 3I). M1 polarized TAMs were also identified with selective staining and gating by flow cytometry using CD86 and MHC Class II antibodies (Fig. 3J) confirming that knockdown of *ELANE* expression in pancreatic cancer induces plasticity of TAMs to increase the number of M1-polarized or anti-cancer macrophages. Similarly, to identify arginase-1 positive N2-polarized

neutrophils distinct from macrophages, dissociated tumors were subjected to flow cytometry after being co-stained with arginase-1 and Ly6G antibodies to gate selectively for neutrophils. The mean number of intratumoral N2-polarized neutrophils in the *ELANE*-KD tumors was significantly lower than the number of neutrophils in the wild-type tumors (Fig. 3K). To evaluate if *ELANE*-KD also influences plasticity of neutrophils, we performed immunohistochemistry on the tumors with FasR and showed that *ELANE*-KD reprograms neutrophils from the N2 polarization to the N1 phenotype (Fig. 3L).

To demonstrate the spatial relationship between the macrophages and the cancer epithelial cells, tissues were reacted with an anti-human pan-cytokeratin antibody and macrophages were stained with CD68, F4/80 and CD206. Representative AsPC-1 tumors clearly distinguish between the cancer cells and immune cells (Supplementary Fig. S2).

#### **Proglumide decreases cytokines by interrupting the cross-communication between G-protein-coupled receptors (GPCRs)**

Above, we demonstrated how pancreatic cancer cells influence the polarity of TAMs by releasing neutrophil elastase in exosomes. We also found that macrophages release factors into media during co-culture that influence cancer cells by increasing the expression of *ELANE* mRNA and promoting cancer growth. CCL2 and CCL20 are two major chemokines released by macrophages that are responsible in part for promoting cancer growth. Since proglumide treatment decreased the growth of the AsPC-1 spheroids in the presence of M2-polarized macrophage supernatant spent media or co-culture, we hypothesized that proglumide may be interrupting the cross-communication of chemokines and cancer cells. To understand the mechanism by which proglumide interrupts the action of chemokines, we treated AsPC-1 and PANC-1 pancreatic cancer cells in culture with chemokines CCL20 and CCL2. First, we confirmed the presence of surface receptors (CCK-BR, CCR6, and CCR2) on the AsPC-1 pancreatic cancer cells using flow cytometry (Fig. 4A, B, and C), respectively. A cartoon model of the experiments is shown where we separately treated AsPC-1 cancer cells in culture with CCL20 (Fig. 4D) or CCL2 (Fig. 4E) with and without proglumide to measure the release of cytokines (TNF $\alpha$  and interferon-gamma (IFN $\gamma$ ), respectively. Since proglumide is a CCK-B receptor antagonist that selectively interacts with the CCK-BR and not CCR6, proglumide would not be expected to affect the release of TNF $\alpha$  from CCR6 positive cells. As expected, proglumide treatment alone did not affect TNF $\alpha$  levels compared to unstimulated cells. Treatment of the cells with CCL20 that interacts solely with

CCR6 resulted in increased detection of TNF $\alpha$  levels in the media (Fig. 4D, graphic red column) compared to unstimulated cells. However, when the cells were treated with both proglumide and CCL20, the release of TNF $\alpha$  was significantly decreased suggesting that blockade of the CCK-BR influenced the intracellular production or release of TNF $\alpha$  after stimulation of the CCR6 receptor via CCL20. Next, AsPC-1 cells treated with the chemokine CCL2 released IFN $\gamma$  into the culture media by its interaction with CCR2, and this effect was blocked by proglumide (Fig. 4E, right).

Similarly, the receptors in human PANC-1 cells were confirmed by flow cytometry for CCK-B receptors (Fig. 4F) and for CCR2 chemokine receptors (Fig. 4G). CCR6 has surface receptor has been well-characterized in PANC-1 cancer cells [42]. To confirm proglumide treatment inhibits cytokine release in a second pancreatic cancer cell line, CCR2-expressing PANC-1 cells were treated *in vitro* with CCL2 with and without proglumide (Fig. 4H). CCL2 induced the release of IFN $\gamma$  into the growth media and this effect was blocked by concomitant treatment with the CCK-BR antagonist proglumide. Since proglumide monotherapy did not affect IFN $\gamma$  levels, the reduction of IFN $\gamma$  by concomitant proglumide treatment with CCL2 suggests that blockade of the CCK-BR decreases cytokine levels by interrupting the intracellular production of CCR2 or the release of IFN $\gamma$ . To confirm that treatment with proglumide decreased intracellular cytokine content rather than just preventing the release of cytokines, we also measured intracellular cytokine levels with flow cytometry. PANC-1 cells were treated with CCL20 with and without proglumide after treatment with Brefeldin-A to block secretion of TNF $\alpha$  and allow accumulation in the Golgi apparatus. Figure 4I shows the shift in intracellular TNF $\alpha$  in PANC-1 cells that were treated with CCL20 and proglumide. These results suggest that the intracellular cytokine concentration is decreased with proglumide treatment. Cholecystikinin receptors and chemokine receptors are both GPCRs, and GPCRs are known to "cross-talk" or influence the action of each other, either by sensitizing or desensitizing the intracellular signaling or downstream pathways of each other or by forming 'heterodimers' [43] to mediate the effects. Our results demonstrate how blocking the CCK-BR with proglumide also downregulates the intracellular cytokines after activation of chemokine receptors.

#### **Proglumide treatment decreases intracellular signaling pathways that regulate cancer growth**

Not only does blockade of the CCK-BRs on pancreatic cancer cells decrease cytokine and neutrophil elastase release in the TME influencing the immune cell signature, but proglumide alone in cell culture decreases AsPC-1 cell proliferation by silencing important intracellular

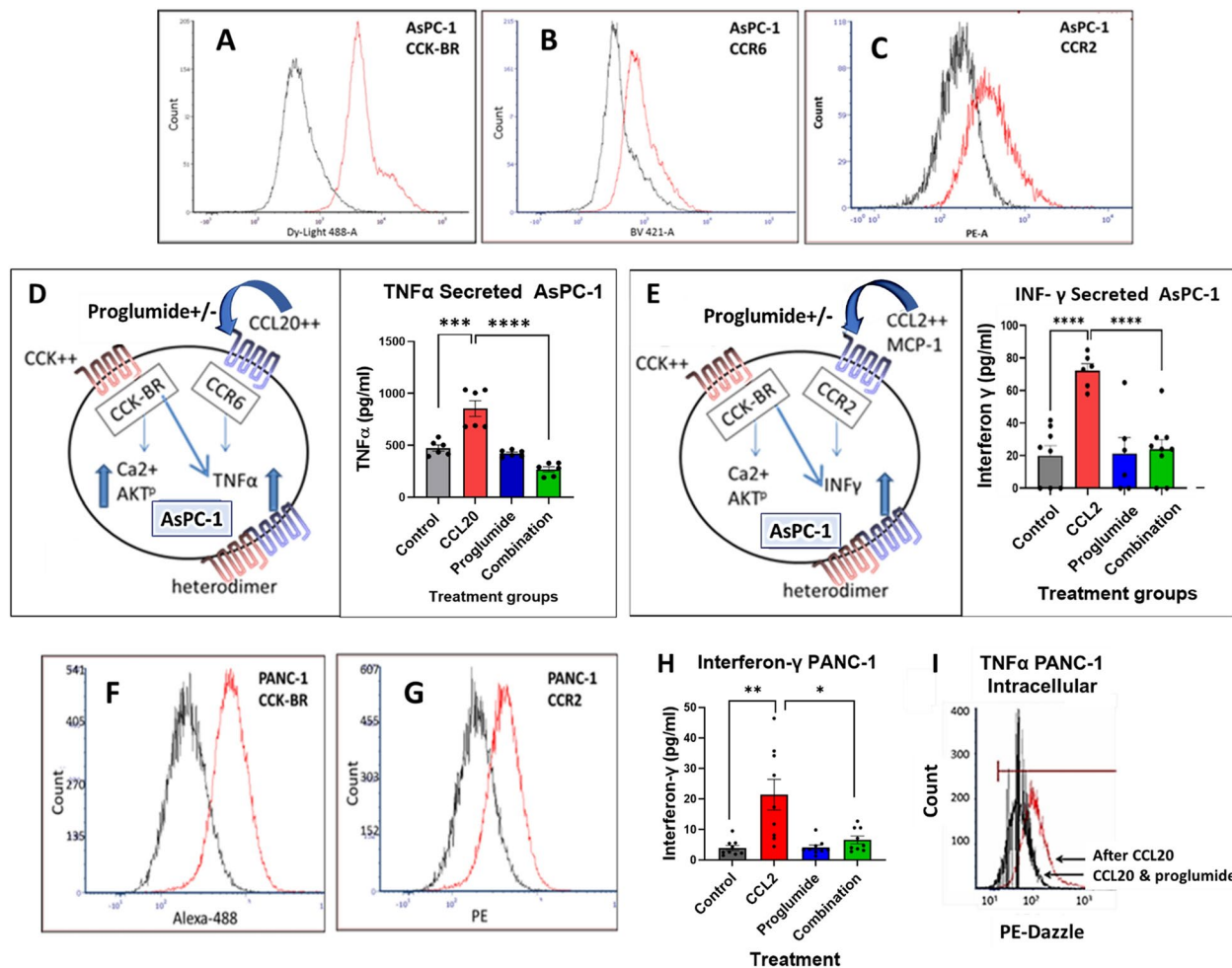


**Fig. 4** Cross-communication between G-protein coupled receptors on pancreatic cancer cells. **A** Human AsPC-1 pancreatic cancer cells exhibit CCK-B receptors on the surface membrane by flow cytometry. **B** AsPC-1 cells have CCR6 chemokine surface receptors. **C** AsPC-1 cancer cells also express CCR2 chemokine receptors by flow cytometry. **D** Representation of the suggested interplay between CCK-BR and CCR6 and expected effects of proglumide and CCL20 on the G-protein coupled receptors downstream signaling activity. On the right, a bar graph with mean and SEM capturing TNF $\alpha$  levels measured in the media of AsPC-1 cells by ELISA in untreated control cells and after incubation with CCL20 and proglumide as single agents and in combination. **E** Representation of the suggested interplay between CCK-BR and CCR2 and the effect of proglumide and CCL20 on the G-protein coupled receptors downstream signaling activity. On the right, a bar graph with mean and SEM capturing INF $\gamma$  levels measured in the media of AsPC-1 cells by ELISA in untreated control cells and after incubation with CCL20 and proglumide as single agents and in combination. **F** Human PANC-1 pancreatic cancer cells exhibit CCK-B receptors on the surface membrane by flow cytometry. **G** Human PANC-1 pancreatic cancer cells exhibit CCR2 receptors on the surface membrane by flow cytometry. **H** Bar graphs with mean and SEM capturing TNF $\alpha$  levels measured in the media of PANC-1 cells by ELISA in untreated control cells and after incubation with CCL20 and proglumide as single agents and in combination. **I** Histogram capturing intracellular TNF $\alpha$  production in PANC-1 cells after treatment with CCL20 and Golgi block with and without proglumide measured by flow cytometry. Assays were performed in replicates X3. Statistical analysis performed by Student's t-test and a Bonferroni correction for multiple comparisons to controls: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.005$ ; \*\*\*\* $P < 0.0001$ .

signaling pathways. Analysis of the RNA sequencing data comparing mRNA expression levels in cancer spheroids alone or with proglumide was evaluated using Ingenuity Pathway Analysis software. The mutant Kras signaling in pancreatic cancer regulates the downstream inositol signaling pathway with activation of PI3K [44]. Specifically, treatment of AsPC-1 spheroids with proglumide resulted in the downregulation of the PI3K signaling pathway (Fig. 5A); a critically important pathway involved in tumor growth. *STAT5* mRNA expression is also downregulated

by proglumide. *STAT5* is a downstream effector of oncogenic Kras that plays a key role in pancreatic cancer progression, and is associated with a poorer prognosis [45]. IPA computer analysis revealed that the *CD247* gene is upregulated by proglumide (Fig. 5A). *CD247* is a T-cell surface glycoprotein that plays a crucial role in the structure, expression, and function of T-cell receptors and NK cell-activating receptors. In the context of pancreatic cancer, *CD247* expression is often downregulated in T cells resulting in an immunosuppressive state with

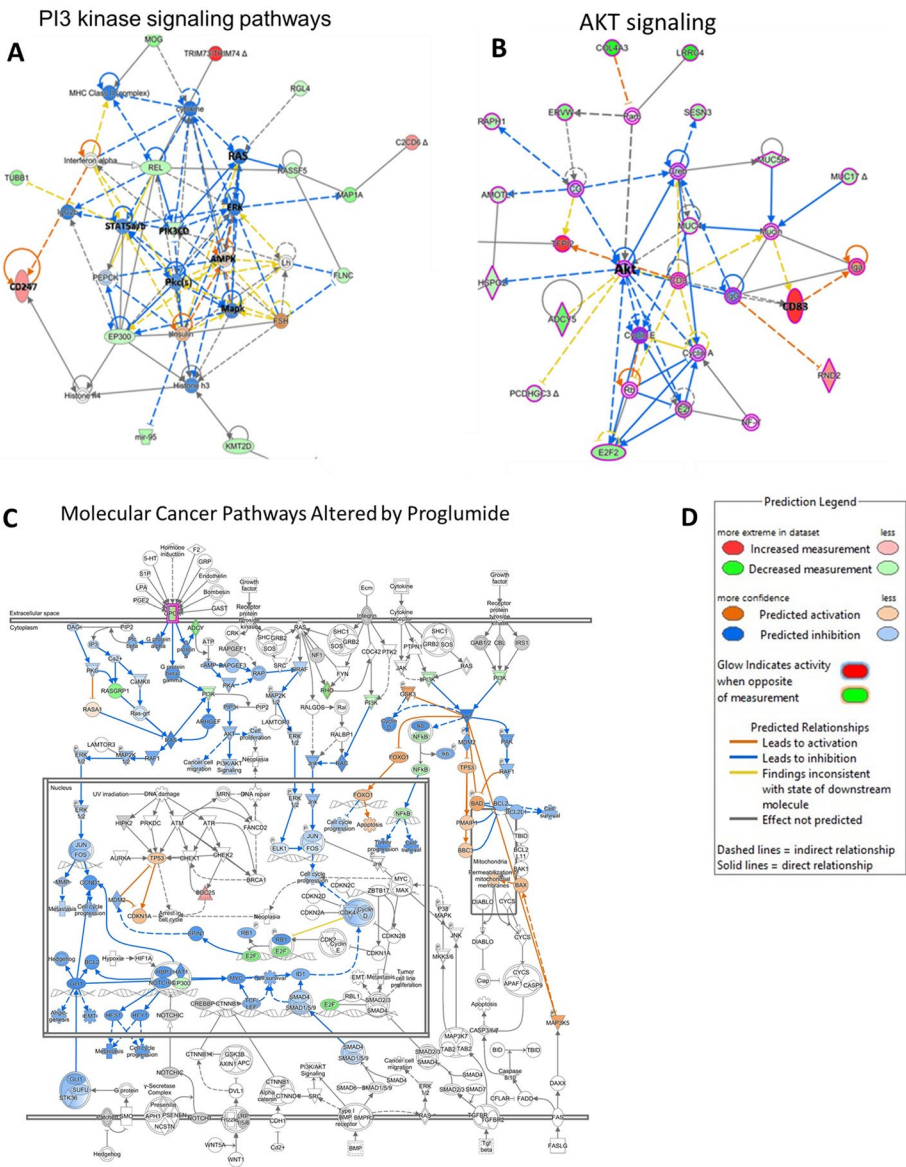


**Fig. 5** Molecular cancer signaling pathways effected by proglumide. **A** PI3 Kinase pathway is downregulated by proglumide treatment of human AsPC-1 spheroids by RNA sequencing. **B** AKT signaling pathway is downregulated by proglumide treatment of AsPC-1 spheroids in culture by RNA sequencing. **C** Diagram overview is shown of the important signaling pathways that are downregulated with proglumide treatment in pancreatic cancer. **D** Legend showing the colors of the circles that correlate with the up- or down-regulated genes. Red donates increased measurement; green denotes decreased measurement; orange predicts activation; and blue predicts inhibition. Solid lines represent direct interaction with other genes. Graphs designed by Qiagen Ingenuity Pathway Analysis software

impaired T-cell function. *CD247* expression is increased in the RNAseq data from spheroids treated with proglumide suggesting that T-cell function may improve with proglumide. Proglumide also decreased the expression of genes in the *AKT* signaling pathway (Fig. 5B) and *NFκB* pathway (Supplementary Fig. S3). Confirmation of proglumide’s action on these genes includes that these same signaling pathways were decreased by the RPPA analysis in the cancer spheroids. An overview of the complex intracellular pathways mechanistically altered by proglumide treatment is shown in Fig. 5C including the

downregulation of *Kras*, *AKT*, and *PI3K*. Importantly, we show that proglumide therapy upregulates tumor suppressor genes *TP53*, *FOXO1*, and *GSK3*. The proapoptosis genes *BAD* and *BAX* are also upregulated with proglumide therapy. The IPA pathway analysis confirms that proglumide treatment of pancreatic cancer cells downregulates proliferative genes and oncogenic signaling pathways and upregulates genes involved with apoptosis and tumor suppression.

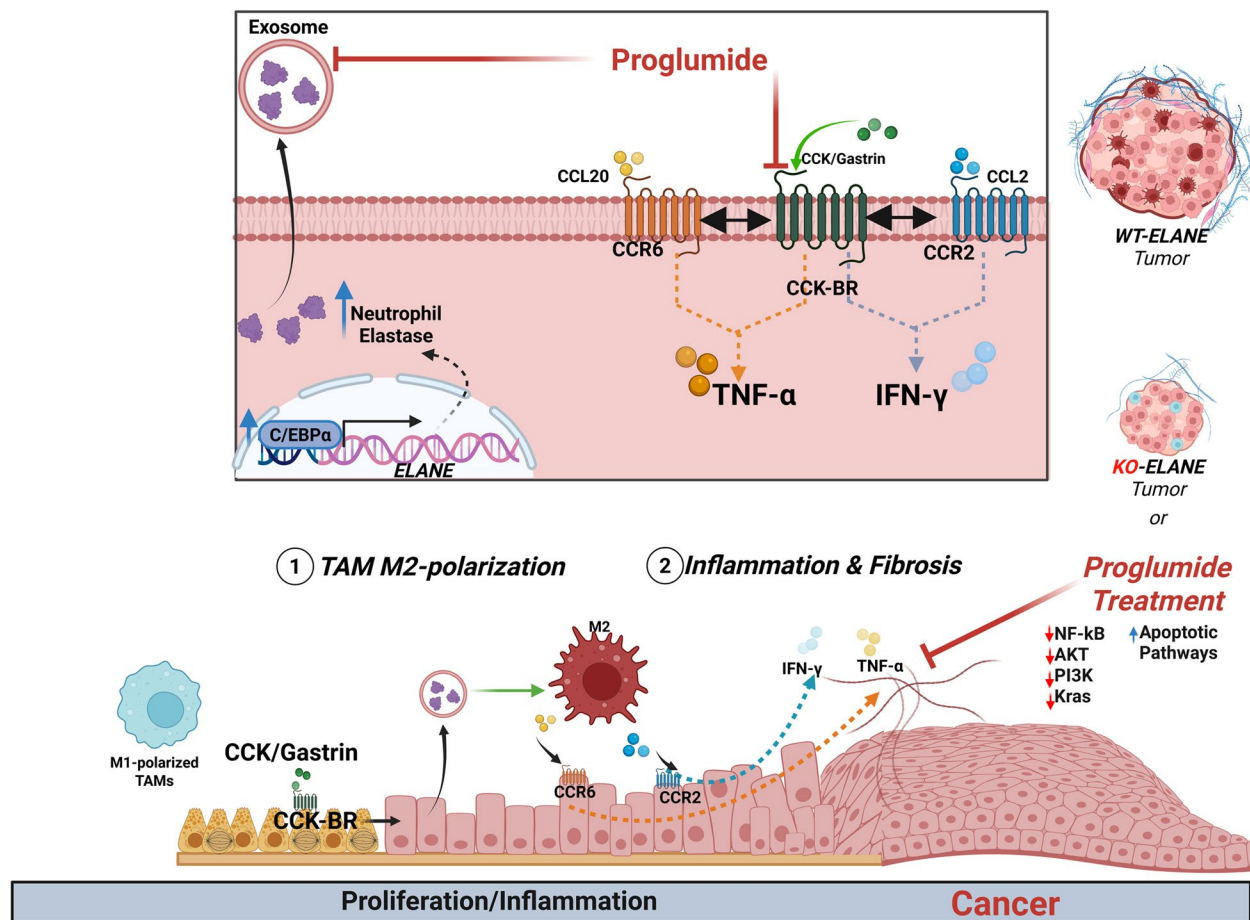


**Fig. 6** Summary of how proglumide interrupts cross-communication pathways in pancreatic cancer. During pancreatic carcinogenesis, the CCK-BR becomes over-expressed on pancreatic cancer cells. The upregulation of *ELANE* mRNA expression through the C/EBP $\alpha$  transcription factor occurs when M0 or M $\Phi$  macrophages are in close proximity to pancreatic cancer cells. In response, neutrophil elastase is released in tumor-derived extracellular vesicles that induce the polarization of macrophages to the pro-tumor M2-phenotype. The M2-polarized TAMs release chemokines CCL20 and CCL2 into the media resulting in increased TNF $\alpha$  and INF $\gamma$ , respectively from cancer cells. These inflammatory cytokines activate signaling pathways like NF- $\kappa$ B and PI3K/Akt to promote tumor growth and epithelial to mesenchymal transition. Proglumide blocks the CCK-BR on cancer cells to downregulate the release of the inflammatory cytokines by cross-communicating intracellularly with the chemokine GPCRs. Proglumide also blocks the release of tumor-derived extracellular vesicles loaded with neutrophil elastase. Direct downregulation of *ELANE* mRNA expression in pancreatic cancer cells decreases tumor growth and induces plasticity of the pro-cancer M2 TAMs to the anti-cancer M1 phenotype in the TME. Blockade CCK-BR signaling with proglumide results in the increased expression of apoptosis pathways and decrease in the oncogenic or proliferative pathways either directly or by decreasing inflammatory cytokines. Created in BioRender. Drda, J. (2025) <https://BioRender.com/io9d6en>

## Discussion

It is well-established that pancreatic cancer cells communicate with immune cells in the tumor microenvironment rendering the TME immunosuppressive and favoring conditions to promote tumor growth. Researchers have been trying strategies to interfere with these communication pathways without harming the normal cells in the host or normal pancreas. The novel finding of this work is that a compound classified as a CCK receptor antagonist, proglumide, can interrupt cross-communication between cancer cells and immune cells in the TME by several mechanisms (Fig. 6). One technique used by cancer cells to manipulate monocytes to become the pro-cancer phenotype involves the upregulation of *ELANE* expression

in the cancer cells and the release of neutrophil elastase in extracellular vesicles. A striking finding of this work is that we demonstrated that treatment of pancreatic cancer cells with proglumide interrupted the signal sent by AsPC-1 cells to the macrophages. Proglumide completely blocked the immunoreactivity for neutrophil elastase in the exosomes. *ELANE* mRNA expression is regulated by a crucial transcription factor, C/EBP $\alpha$  that is a member of the CCAAT/enhancer-binding protein (C/EBP) family. The C/EBP family consists of six structurally and functionally homologous transcription factors: C/EBP $\alpha$ , C/EBP $\beta$ , C/EBP $\gamma$ , C/EBP $\delta$ , C/EBP $\epsilon$ , and CHOP [46]. Of these, C/EBP $\alpha$  is a crucial transcription factor for the differentiation of granulocytes, the cell lineage that includes

neutrophils and monocytes. C/EBP $\alpha$  binds to the *ELANE* gene promoter to initiate transcription. The C/EBP $\alpha$  is not carried by exosomes, but a long non-coding RNA associated with it, CEBPA-AS1, is packaged within exosomes. Piao et al. [47] found in gastric cancer that CEBPA-AS1-containing exosomes promoted cell proliferation, inhibited apoptosis, and induced cancer progression, indicating that exosomal CEBPA-AS1 is involved in cell-to-cell communication.

Neutrophil elastase, the protein coded by the *ELANE* gene, is not solely produced by neutrophils but also by a variety of immune cells, including myeloid-derived suppressor cells (MDSCs) and macrophages [48]. In our study, we found that the source of neutrophil elastase was from the cancer cells and that the macrophages had some *ELANE* expression but much lower than the cancer cells. Neutrophil elastase has been implicated in the progression and metastasis of pancreatic cancer by several mechanisms. It degrades E-cadherin, thus facilitating tumor cell detachment and migration, and can stimulate proliferative pathways by extracellular transactivation of membrane receptors such as epidermal growth factor receptor (EGFR) and toll-like receptor 4 (TLR4), inducing mitogen-activated protein kinase (MAPK) signaling and downstream effects [49]. Neutrophil elastase is an integral component of neutrophil extracellular traps (NETs) that promote tumor growth and metastasis [50]. For these reasons, strategies have been proposed to target neutrophil elastase in cancer therapy [51]. Our study shows that mRNA downregulation of *ELANE* renders the pancreatic cancer TME less immunosuppressive and results in decreased tumor growth. But genetic manipulation of *ELANE* expression in the host could be detrimental since neutrophil elastase is also important for combating infections. However, strategies that control the activation of neutrophil elastase in tumors with medications like proglumide may be beneficial in cancer therapeutics.

Although the focus of this work was on the macrophages and their interaction with pancreatic cancer, there are many other components and cell types in the TME. In particular, the neutrophil has recently been recognized as playing a key role in the pancreatic cancer TME including its interaction with macrophages. Both TAMs and neutrophils have immunosuppressive properties in the pancreatic TME that promote tumor growth and metastases [52]. The number of infiltrating neutrophils in a pancreatic tumor and the neutrophil-to-lymphocyte ratio predict an adverse outcome in the survival of pancreatic cancer [53]. Similar to the macrophages, neutrophils have been shown to exhibit plasticity and can change polarization from the anti-cancer N1 to the pro-cancer N2 phenotype [54]. In our work, we showed that the number of tumor-infiltrating N2-polarized neutrophils decreased in

AsPC-1 pancreatic tumors with *ELANE*-KD supporting the intercommunication between cells in the TME.

Another mechanism that proglumide uses to interrupt communication between cells in the TME was by blocking the crosstalk between GPCRs (i.e., chemokine and CCK receptors). We showed that blockade of CCK-BR signaling with proglumide reduced inflammatory cytokines by interfering with intracellular crosstalk between GPCRs. Proglumide antagonism at the CCK-BR cross-communicated intracellularly to the chemokine surface receptors decreasing the response to CCL20 and CCL2 by inhibiting the release of inflammatory cytokines TNF $\alpha$  and IFN $\gamma$ , respectively. Strategies to interrupt the CCL2-CCR2 axis in the clinic have been attempted. PF-04136309 is an oral CCR2 antagonist which blocks the CCL2-CCR2 chemokine axis but unfortunately failed in clinical trials due to pulmonary toxicity [55]. In relation to our current work, we showed that the GPCR, CCR2 crosstalks with the CCK-BR and that proglumide indirectly blocks the action of the CCL2-CCR2 signaling and the release of IFN $\gamma$ . In addition to cytokine production from stroma and immune cells, tumor-induced cytokine production in the TME has been shown to promote and accelerate cancer development [56]. In the pancreatic cancer TME, TNF $\alpha$  has been shown to function as a tumor promoter, increasing growth of cancer cells [57]. IFN $\gamma$  has been shown to promote epithelial to mesenchymal transition in pancreatic tumors [58]. Therefore, methods to decrease the release of these cytokines in the TME would be beneficial and inhibit cancer growth.

Lastly, our results show that co-culture of AsPC-1 spheroids with macrophages significantly increases the expression of oncogene signaling pathways by RNAseq and RPPA. Proglumide therapy reverses the activation of the oncogenic pathways thus, this compound has direct anti-cancer properties apart from its interaction with the immune compartment in PDAC. Blockade of the CCK-BR signaling pathway not only decreases oncogenic pathways but also increases apoptosis pathways and tumor suppressor genes. These features should favor the inhibition of cancer growth and perhaps improve efficacy of cancer therapeutics.

A shortcoming of our animal studies is that we used immune deficient mice since we were analyzing the growth of human tumors. Although the athymic nude mice have normal neutrophils and macrophages they lack T-cells; therefore, we could not evaluate the effects of *ELANE* on T-cells in the TME. We have previously shown in immune competent mice bearing syngeneic murine pancreatic tumors, that proglumide therapy also plays a role in remodeling the fibrosis of the TME and increasing the influx of CD8 $^{+}$  T cells [12]. With the decreased tumoral fibrosis not only can T-cells penetrate

the tumors but uptake of chemotherapy [12] and immune checkpoint inhibitors is significantly improved.

Proglumide is an older drug that was developed many years ago for the treatment of peptic ulcer disease, but commercialization was halted with the approval of more potent proton pump inhibitors. However, proglumide was tested in over 600 subjects and found to have a broad safety profile. Our team has completed a safety and dose—finding study of proglumide in human subjects with steatohepatitis (NCT#04152473) [59] and found it was well tolerated at a dose of 1200 mg/day orally. Recently, we also completed a Phase 1 trial using proglumide with chemotherapy in patients with metastatic pancreatic cancer (NCT5827055, Abstract AACR 2025). In this clinical trial, pre-treatment and on-treatment tumor biopsies from patients with metastatic pancreatic cancer confirmed that proglumide decreased the M2-polarized TAMs in the TME.

In conclusion, new approaches are desperately needed for pancreatic cancer therapy. Proglumide offers a novel strategy to reprogram the host TME and render it more susceptible to treatment. Since proglumide is orally bioavailable and has a broad safety profile its use as an adjunctive therapy to pancreatic cancer has potential. Due to its ability to interrupt several pathways of communication between the cancer cells and the immune cells in the TME, further studies are warranted in pancreatic cancer and other cancers.

#### Abbreviations

|                |                                                                |
|----------------|----------------------------------------------------------------|
| CCK            | Cholecystokinin                                                |
| CCK-BR         | Cholecystokinin-B receptor                                     |
| CCL2           | Chemokine (C–C motif) ligand 2                                 |
| CCL20          | Chemokine (C–C motif) ligand 20                                |
| CCR2           | Receptor or CCL2                                               |
| CCR6           | Receptor for CCL20                                             |
| DEGs           | Differentially expressed genes                                 |
| EGFR           | Epidermal growth factor receptor                               |
| <b>ELANE</b>   | Gene that codes for neutrophil elastase                        |
| ELISA          | Enzyme-linked immunosorbent assay                              |
| ERK            | Extracellular signal-Regulated Kinase                          |
| EVs            | Extracellular vesicles or exosomes                             |
| GPCR           | G-protein coupled receptor                                     |
| IFN $\gamma$   | Interferon gamma                                               |
| IPA            | Ingenuity Pathway Analysis                                     |
| MTT            | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide   |
| NETs           | Neutrophil extracellular traps                                 |
| NF- $\kappa$ B | Nuclear Factor kappa-light-chain-enhancer of activated B cells |
| PAK            | P21-activated kinase                                           |
| PDAC           | Pancreatic ductal adenocarcinoma                               |
| PI3K           | Phosphoinositide 3-kinase                                      |
| PKC            | Protein kinase C                                               |
| PMA            | Phorbol myristate acetate                                      |
| RPPA           | Reverse Phase Protein Array                                    |
| STAT3          | Signal Transducer and Activator of Transcription 3             |
| TAMs           | Tumor associated macrophages                                   |
| TME            | Tumor microenvironment                                         |
| TNF $\alpha$   | Tumor necrosis factor alpha                                    |

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12964-026-02676-8>.

Supplementary Material 1.

#### Acknowledgements

Acknowledgments: We appreciate the staff in the Department of Comparative Medicine with their care for the mice and the staff from the Lombardi Core Shared resources for assistance with histology and flow cytometry.

#### Authors' contributions

JPS and MP conceived the study. JPS, MM, JK, AN, JD, MDG, HC, WC, AG, EB, NS, JD and MP performed the experiments; WC, QW, AA, WD, and HF analyzed the data; JPS and MP supervised the experiments; JPS and MP wrote the manuscript and provided funding; All the authors reviewed the final version.

#### Funding

Funding for this research was supported by an NIH/NCI grant (R01CA277680) to JPS and MP. A grant from the American Cancer Society (ACS) Diversity in Cancer Research (DICR) awards DICR INTR-23- supported JK. Funding from the Summer Undergraduate Research Fellowships from the American Physiological Society supported JD. A shared core facilities grant to Lombardi Comprehensive Cancer Center from NIH/NCI grant P30-CA051008 for histology and flow cytometry. We appreciate the support from the Georgetown University Master's Graduate program in Biochemistry and Molecular Biology for MM.

#### Data availability

Data is available in the supplementary files. Raw data for RNA sequencing is available on the Zenodo website at [<https://doi.org/10.5281/zenodo.1603806>] (<https://doi.org/10.5281/zenodo.1603806>).

#### Declarations

#### Competing interests

Competing Interests: Georgetown University owns an issued patent for the use of proglumide in pancreatic cancer and Dr Jill Smith is a co-inventor; Mariaelena Pierobon is an inventor of university-assigned patents and patent applications and received royalties from and consulted for TheraLink Technologies. The other authors have no competing interests.

#### Author details

<sup>1</sup>Departments of Medicine, Georgetown University, Washington, DC, USA

<sup>2</sup>Department of Biochemistry and Molecular Biology, Georgetown University, Washington, DC, USA

<sup>3</sup>University of Maryland, College Park, MD, USA

<sup>4</sup>University of Pittsburgh, Pittsburgh, PA, USA

<sup>5</sup>Center for Applied Proteomics and Molecular Medicine/School of Systems Biology, Manassas, VA, USA

<sup>6</sup>Department of Biostatistics, Georgetown University, Washington, DC, USA

<sup>7</sup>Department of Bioengineering, George Mason University, Manassas, VA, USA

Received: 1 August 2025 / Accepted: 11 January 2026

Published online: 16 January 2026

#### References

1. Truong LH, Pauklin S. Pancreatic Cancer Microenvironment and Cellular Composition: Current Understandings and Therapeutic Approaches. *Cancers* (Basel). 2021;13(19):5028. <https://doi.org/10.3390/cancers13195028>.
2. Chang CH, Pauklin S. Extracellular vesicles in pancreatic cancer progression and therapies. *Cell Death Dis*. 2021;12:973.
3. Panda A, Falasca M, Ragunath K. Extracellular vesicles in pancreatic cancer: a new era in precision medicine. *Transl Gastroenterol Hepatol*. 2024;9:29.

4. Honselmann KC, Finetti P, Birnbaum DJ, Monsalve CS, Wellner UF, Begg SKS, et al. Neoplastic-stromal cell cross-talk regulates matrisome expression in pancreatic cancer. *Mol Cancer Res*. 2020;18:1889–902.
5. Geleta B, Tout FS, Lim SC, Sahni S, Jansson PJ, Apte MV, et al. Targeting Wnt/tenascin C-mediated cross talk between pancreatic cancer cells and stromal cells via activation of the metastasis suppressor NDRG1. *J Biol Chem*. 2022;298:101608.
6. Samaniego R, Gutierrez-Gonzalez A, Gutierrez-Seijo A, Sanchez-Gregorio S, Garcia-Gimenez J, Mercader E, et al. Ccl20 expression by tumor-associated macrophages predicts progression of human primary cutaneous melanoma. *Cancer Immunol Res*. 2018;6(3):267–75.
7. Rubie C, Frick VO, Ghadjar P, Wagner M, Grimm H, Vicinus B, et al. CCL20/CCR6 expression profile in pancreatic cancer. *J Transl Med*. 2010;8:45.
8. Kadomoto, S.; Izumi, K.; Mizokami, A. Roles of CCL2-CCR2 Axis in the Tumor Microenvironment. *Int J Mol Sci*. 2021;22.
9. Li X, He G, Liu J, Yan M, Shen M, Xu L, et al. CCL2-mediated monocytes regulate immune checkpoint blockade resistance in pancreatic cancer. *Int Immunopharmacol*. 2022;106:108598.
10. Mantovani A, Allavena P, Marchesi F, Garlanda C. Macrophages as tools and targets in cancer therapy. *Nat Rev Drug Discov*. 2022;21:799–820.
11. Wang S, Wang J, Chen Z, Luo J, Guo W, Sun L, et al. Targeting M2-like tumor-associated macrophages is a potential therapeutic approach to overcome antitumor drug resistance. *NPJ Precis Oncol*. 2024;8:31.
12. Malchiodi ZX, Cao H, Gay MD, Safronenka A, Bansal S, Tucker RD, Weinberg BA, Cheema A, Shivapurkar N, Smith JP. Cholecystokinin Receptor Antagonist Improves Efficacy of Chemotherapy in Murine Models of Pancreatic Cancer by Altering the Tumor Microenvironment. *Cancers (Basel)*. 2021;13(19):4949.
13. Nadella S, Ciofoaia V, Cao H, Kallakury B, Tucker RD, Smith JP. Cholecystokinin receptor antagonist therapy decreases inflammation and fibrosis in chronic pancreatitis. *Dig Dis Sci*. 2020;65:1376–84.
14. Tucker RD, Ciofoaia V, Nadella S, Gay MD, Cao H, Huber M, et al. A Cholecystokinin Receptor Antagonist Halts Nonalcoholic Steatohepatitis and Prevents Hepatocellular Carcinoma. *Dig Dis Sci*. 2020;65:189–203.
15. Gay MD, Cao H, Shivapurkar N, Dakshanamurthy S, Kallakury B, Tucker RD, Kwagyan J, Smith JP. Proglumide Reverses Nonalcoholic Steatohepatitis by Interaction with the Farnesoid X Receptor and Altering the Microbiome. *Int J Mol Sci*. 2022;23(3):1899.
16. Liu B, Jia Y, Ma J, Wu S, Jiang H, Cao Y, et al. Tumor-associated macrophage-derived CCL20 enhances the growth and metastasis of pancreatic cancer. *Acta Biochim Biophys Sin*. 2016;48:1067–74.
17. Sanford DE, Belt BA, Panni RZ, Mayer A, Deshpande AD, Carpenter D, et al. Inflammatory monocyte mobilization decreases patient survival in pancreatic cancer: a role for targeting the CCL2/CCR2 axis. *Clin Cancer Res*. 2013;19:3404–15.
18. Sierra-Filardi E, Nieto C, Dominguez-Soto A, Barroso R, Sanchez-Mateos P, Puig-Kroger A, et al. Ccl2 shapes macrophage polarization by GM-CSF and M-CSF: identification of CCL2/CCR2-dependent gene expression profile. *J Immunol*. 2014;192:3858–67.
19. Steinberg E, Orehov N, Tischenko K, Schwob O, Zamir G, Hubert A, Manevitch Z, Benny O. Rapid Clearing for High Resolution 3D Imaging of Ex Vivo Pancreatic Cancer Spheroids. *Int J Mol Sci*. 2020;21(20):7703.
20. Foty R. A simple hanging drop cell culture protocol for generation of 3D spheroids. *J Vis Exp*. 2011(51):2720.
21. Blanchard CE, Gomeiz AT, Avery K, Gazzah EE, Alsubaie AM, Sikaroodi M, et al. Signaling dynamics in coexisting monoclonal cell subpopulations unveil mechanisms of resistance to anti-cancer compounds. *Cell Commun Signal*. 2024;22:377.
22. Pierobon M, Ramos C, Wong S, Hodge KA, Aldrich J, Byron S, et al. Enrichment of PI3K-AKT-mTOR Pathway Activation in Hepatic Metastases from Breast Cancer. *Clin Cancer Res*. 2017;23:4919–28.
23. Baldelli, E.; Calvert, V.; Hodge, A.; VanMeter, A.; Petricoin, E.F., III; Pierobon, M. Reverse Phase Protein Microarrays. *Methods Mol Biol*. 2017;1606:149–169.
24. Signore M, Manganelli V, Hodge A. Antibody validation by western blotting. *Methods Mol Biol*. 2017;1606:51–70.
25. Pin E, Federici G, Petricoin EF III. Preparation and use of reverse protein microarrays. *Curr Protoc Protein Sci*. 2014;75:27.
26. Baxter EW, Graham AE, Re NA, Carr IM, Robinson JI, Mackie SL, et al. Standardized protocols for differentiation of THP-1 cells to macrophages with distinct M(IFN $\gamma$ +LPS), M(IL-4) and M(IL-10) phenotypes. *J Immunol Methods*. 2020;478:112721.
27. Nascimento CR, Rodrigues Fernandes NA, Gonzalez Maldonado LA, Rossa JC. Comparison of monocytic cell lines U937 and THP-1 as macrophage models for in vitro studies. *Biochem Biophys Rep*. 2022;32:101383.
28. Ishiguro T, Ohata H, Sato A, Yamawaki K, Enomoto T, Okamoto K. Tumor-derived spheroids: relevance to cancer stem cells and clinical applications. *Cancer Sci*. 2017;108:283–9.
29. Shi X, Yang J, Liu M, Zhang Y, Zhou Z, Luo W, et al. Circular RNA ANAPC7 Inhibits Tumor Growth and Muscle Wasting via PHLPP2-AKT-TGF- $\beta$  Signaling Axis in Pancreatic Cancer. *Gastroenterology*. 2022;162:2004–17.
30. Borden KLB. The Nuclear Pore Complex and mRNA Export in Cancer. *Cancers (Basel)*. 2020;13(1):42.
31. Tang X, Jiang N, Kou Y, Cheng S, Yan F. PLA2G4F is a metabolic checkpoint in triple-negative breast cancer: insights from multiple omics analysis and experiments. *Mol Ther Oncolytics*. 2025;33:200963.
32. Yang Y, Gao L, Weng NN, Li JJ, Liu JL, Zhou Y, et al. Identification of novel molecular therapeutic targets and their potential prognostic biomarkers among kinesin superfamily of proteins in pancreatic ductal adenocarcinoma. *Front Oncol*. 2021;11:708900.
33. Al-Taei KMK, Zepp M, Berger I, Berger MR, Adwan H. Pancreatic carcinoma cells colonizing the liver modulate the expression of their extracellular matrix genes. *Genes Cancer*. 2018;9:215–31.
34. Yang X, Wu Q, Wu F, Zhong Y. Differential expression of COL4A3 and collagen in upward and downward progressing types of nasopharyngeal carcinoma. *Oncol Lett*. 2021;21:223.
35. McDonald MF, Curry RN, O'Reilly I, Lozzi B, Cervantes A, Lee ZF, et al. Tumor Expression of CD83 Reduces Glioma Progression and Is Associated with Reduced Immunosuppression. *Cancer Res Commun*. 2024;4:3209–23.
36. Chen H, Liang W, Zheng W, Li F, Pan X, Lu Y. A novel telomere-related gene prognostic signature for survival and drug treatment efficiency prediction in lung adenocarcinoma. *Aging (Albany NY)*. 2023;15:7956–73.
37. Mehner C, Miller E, Khauv D, Nassar A, Oberg AL, Bamlet WR, et al. Tumor cell-derived MMP3 orchestrates Rac1b and tissue alterations that promote pancreatic adenocarcinoma. *Mol Cancer Res*. 2014;12:1430–9.
38. Nuche-Berenguer B, Jensen RT. Gastrointestinal hormones/neurotransmitters and growth factors can activate P21 activated kinase 2 in pancreatic acinar cells by novel mechanisms. *Biochim Biophys Acta*. 2015;1853:2371–82.
39. Nadella S, Burks J, Huber M, Wang J, Cao H, Kallakury B, et al. Endogenous gastrin collaborates with mutant KRAS in pancreatic carcinogenesis. *Pancreas*. 2019;48:894–903.
40. Mao H, Zhao X, Sun SC. NF-kappaB in inflammation and cancer. *Cell Mol Immunol*. 2025;22:811–39.
41. Wu N, Xu XF, Xin JQ, Fan JW, Wei YY, Peng QX, et al. The effects of nuclear factor-kappa B in pancreatic stellate cells on inflammation and fibrosis of chronic pancreatitis. *J Cell Mol Med*. 2021;25:2213–27.
42. Kimsey TF, Campbell AS, Albo D, Wilson M, Wang TN. Co-localization of macrophage inflammatory protein-3alpha (Mip-3alpha) and its receptor, CCR6, promotes pancreatic cancer cell invasion. *Cancer J*. 2004;10:374–80.
43. Werry TD, Wilkinson GF, Willars GB. Mechanisms of cross-talk between G-protein-coupled receptors resulting in enhanced release of intracellular Ca<sup>2+</sup>. *Biochem J*. 2003;374:281–96.
44. Murthy D, Attri KS, Singh PK. Phosphoinositide 3-kinase signaling pathway in pancreatic ductal adenocarcinoma progression, pathogenesis, and therapeutics. *Front Physiol*. 2018;9:335.
45. Lin Y, Pu S, Wang J, Wan Y, Wu Z, Guo Y, et al. Pancreatic STAT5 activation promotes Kras(G12D)-induced and inflammation-induced acinar-to-ductal metaplasia and pancreatic cancer. *Gut*. 2024;73:1831–43.
46. Wang W, Xia X, Mao L, Wang S. The CCAAT/enhancer-binding protein family: its roles in MDSC expansion and function. *Front Immunol*. 2019;10:1804.
47. Piao HY, Guo S, Wang Y, Zhang J. Exosomal long non-coding RNA CEBPA-AS1 inhibits tumor apoptosis and functions as a non-invasive biomarker for diagnosis of gastric cancer. *Oncotargets Ther*. 2020;13:1365–74.
48. Dollery CM, Owen CA, Sukhova GK, Krettek A, Shapiro SD, Libby P. Neutrophil elastase in human atherosclerotic plaques: production by macrophages. *Circulation*. 2003;107:2829–36.
49. Lerman I, Hammes SR. Neutrophil elastase in the tumor microenvironment. *Steroids*. 2018;133:96–101.
50. Demkow U. Neutrophil Extracellular Traps (NETs) in Cancer Invasion, Evasion and Metastasis. *Cancers (Basel)*. 2021;13(17):4495.
51. Jia W, Mao Y, Luo Q, Wu J, Guan Q. Targeting neutrophil elastase is a promising direction for future cancer treatment. *Discover Oncol*. 2024;15:167.
52. Pratt HG, Steinberger KJ, Mihalik NE, Ott S, Whalley T, Szomolay B, Boone BA, Eubank TD. Macrophage and Neutrophil Interactions in the Pancreatic Tumor

- Microenvironment Drive the Pathogenesis of Pancreatic Cancer. *Cancers (Basel)*. 2021;14(1):194.
53. Shin K, Jung EK, Park SJ, Jeong S, Kim IH, Lee MA. Neutrophil-to-lymphocyte ratio and carbohydrate antigen 19–9 as prognostic markers for advanced pancreatic cancer patients receiving first-line chemotherapy. *World J Gastrointest Oncol*. 2021;13:915–28.
54. Giese MA, Hind LE, Huttenlocher A. Neutrophil plasticity in the tumor micro-environment. *Blood*. 2019;133:2159–67.
55. Nywening TM, Wang-Gillam A, Sanford DE, Belt BA, Panni RZ, Cusworth BM, et al. Targeting tumour-associated macrophages with CCR2 inhibition in combination with FOLFIRINOX in patients with borderline resectable and locally advanced pancreatic cancer: a single-centre, open-label, dose-finding, non-randomised, phase 1b trial. *Lancet Oncol*. 2016;17:651–62.
56. Kureshi CT, Dougan SK. Cytokines in cancer. *Cancer Cell*. 2025;43:15–35.
57. Alam MS, Gaida MM, Witzel HR, Otsuka S, Abbasi A, Guerin T, et al. Tnfr1 signaling promotes pancreatic tumor growth by limiting dendritic cell number and function. *Cell Rep Med*. 2024;5:101696.
58. Imai D, Yoshizumi T, Okano S, Itoh S, Ikegami T, Harada N, et al. IFN-gamma promotes epithelial-mesenchymal transition and the expression of PD-L1 in pancreatic cancer. *J Surg Res*. 2019;240:115–23.
59. Rabiee A, Gay MD, Shivapurkar N, Cao H, Nadella S, Smith CI, et al. Safety and dosing study of a cholecystokinin receptor antagonist in non-alcoholic steatohepatitis. *Clin Pharmacol Ther*. 2022;112:1271–9.

### Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.