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Vaccination with Polyclonal Antibody Stimulator (PAS) Prevents Pancreatic Carcinogenesis in the KRAS Mouse Model

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ABSTRACT

The incidence of pancreatic cancer is increasing significantly and will soon become the second leading cause of cancer-related deaths in the United States. We have previously shown that the gastrointestinal peptide gastrin, which is only expressed in the fetal pancreas and not in the adult pancreas, is activated during pancreatic carcinogenesis where it stimulates growth in an autocrine fashion. In this investigation, we used transgenic LSL-Kras$^{G12D/+}$; *P48-Cre* mice that develop precancerous pancreatic intraepithelial neoplasia (PanIN) lesions and pancreatic cancer over time. Starting at 3 months of age, mice were either left untreated (control) or were treated with a gastrin-targeted vaccine, polyclonal antibody stimulator (PAS 250 μg) followed by a monthly booster until the mice reached 8 months of age when pancreata were excised, and analyzed by histology for PanIN grade in a blinded fashion. High-grade PanIN-3 lesions were significantly less in PAS-treated mice ($P = 0.0077$), and cancers developed in 33% of the control mice but only in 10% of the PAS-treated mice. Compared with the control mice, fibrosis was reduced by >50%, arginase positive M2 macrophages were reduced by 74%, and CD8$^+$ T cells were increased by 73% in the pancreas extracellular matrix in PAS-treated mice.

Prevention Relevance: PAS vaccination significantly decreased high-grade PanIN lesions and altered the pancreas microenvironment, rendering it less carcinogenic.

Introduction

Cancer prevention is the best approach for improving survival from cancers, and this strategy has proven to be an effective method in several malignancies. Surveillance screening procedures such as colonoscopy or mammography have significantly improved survival from colon (1) and breast cancer (2), respectively. Vaccinations targeted at preventing viral infections such as hepatitis B (3) and the human papillomavirus (4) have also significantly decreased the risk of hepatocellular and cervical cancer. For the prevention of pancreatic cancer, guidelines have been recommended from the International Cancer of the Pancreas Screening (CAPS) Consortium for examination according to age, family history, and germline mutation status (5). This consortium recommends using imaging with MRI/magnetic retrograde cholangiopancreatography (MRCP) and/or endoscopic ultrasound (EUS) in high-risk populations. About 15% of pancreatic cancers arise from pancreatic cystic lesions such as intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasm (MCN) of the pancreas, and guidelines have been published on surveillance of subjects with these cystic lesions using radiographic imaging and endoscopic ultrasound (6). However, the majority (~85%) of pancreatic cancers arises from a histologic lesion called pancreatic intraepithelial neoplasia (PanIN), and these precancerous lesions are not identified on routine radiographic imaging or endoscopic ultrasound (7). With the exception of life style modification such as avoiding smoking and obesity (8), there are no other strategies to prevent pancreatic cancer that develops from the PanIN lesions.

One of the initiatives of the Cancer Moonshot Blue Ribbon panel report was to develop vaccinations for non–viral-mediated cancers (9). A review on immunotherapy and prevention of pancreatic cancer stated that one of the obstacles to overcome in developing a vaccine for pancreatic cancer would be the immunosuppressive microenvironment and paucity of infiltrating T lymphocytes characteristic of this tumor (10). A promising preclinical investigation (11) showed that vaccination with an attenuated intracellular Listeria monocytogenes vaccine targeting mutant KRAS, in Kras$^{G12D/+}$; Trp53$^{R172H/+}$; Pdx-1-Cre (KPC) mice could decrease PanIN progression and improve survival when mice were concomitantly treated with cyclophosphamide and an anti-CD25 antibody to deplete

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immunosuppressive T regulatory cells. Our study supports the idea that pancreatic cancer could potentially be prevented with a vaccine if the tumor microenvironment is also modified rendering it less immunosuppressive.

Gastrin has been shown to stimulate growth of pancreatic cancer in an autocrine fashion (12). In the adult, gastrin is synthesized and secreted from the G cells of the stomach and is not found in the pancreas. During pancreatic carcinogenesis, gastrin (13) and its receptor, the cholecystokinin B receptor (CCK-BR) (14) become expressed in PanINs. Evidence to support the importance of gastrin in pancreatic carcinogenesis was published in a study where gastrin-knockout (KO) transgenic mice (15) were crossed with LSL-Kras$^{G12D/+}$; *P48-Cre* (KC) transgenic mice, and PanIN progression was arrested. Cancer did not occur in the absence of gastrin (16).

Polyclonal antibody stimulator (PAS) is a cancer vaccine comprised of a 9-amino acid epitope derived from the N-terminal sequence of gastrin-17 conjugated to diphtheria toxoid in an oil-based adjuvant. PAS vaccination activates humoral and cellular immunity by eliciting both the production of specific, high-affinity polyclonal anti-gastrin neutralizing antibodies and the activation of T cells (17). In mice bearing pancreatic tumors, vaccination with PAS decreased primary tumor size and prevented metastases (18). A unique feature of PAS found in these murine studies (17) was that the vaccine also altered the tumor microenvironment. PAS increased the influx of CD8$^+$ T lymphocytes and decreased intratumoral immunosuppressive T regulatory cells, thus changing an immunologically "cold" tumor into an immune responsive tumor. In fact, PAS monotherapy modified the immune cells of the tumor microenvironment without the addition of cyclophosphamide or an anti–CD-25 antibody. The purpose of our investigation was to examine whether PAS vaccination could arrest PanIN progression and alter the pancreas tumor microenvironment in LSL-Kras$^{G12D/+}$; *P48-Cre* mice.

Materials and Methods

Vertebrate animals

All studies in mice were performed in an ethical fashion and with the approval of the Georgetown University IACUC. Both male and female littermates from a transgenic LSL-Kras$^{G12D/+}$; *P48-Cre* murine colony were used in this study. This model has previously been characterized (19) and shown to develop precancerous PanIN lesions by 3 months and pancreatic cancer over time. Mice were weaned and genotyped by 30 days of age and those with the LSL-Kras$^{G12D/+}$; *P48-Cre* genotypes were randomized into one of two groups: controls-untreated ($n = 9$) and PAS-treated ($n = 10$).

Treatment

Nineteen age-matched littermates (males and females) were divided into two groups: control/untreated ($n = 9$) and PAS-treated ($n = 10$). There were 2 female and 7 male mice in the control group and 4 female and six male mice in the PAS treatment group. Starting at 3 months of age, the PAS mice received 3 induction doses of PAS 250 μg administered subcutaneously at baseline, week 1 and week 3. Following this induction, the PAS-treated mice received a booster dose of PAS 250 μg subcutaneously every 4 weeks until the mice reached 8 months of age for a total of 4 boosters. All mice, PAS-treated mice and controls, were ethically euthanized at 8 months of age.

Histology and PanIN scoring

For all mice, the pancreas was dissected, fixed in paraformaldehyde, and paraffin embedded. Tissue sections (5 μm) were mounted and stained with hematoxylin and eosin. Histologic sections were scored by a pathologist, blinded to the treatment, for PanIN grade and percentage of PanINs replacing the pancreas. Pancreas sections were scored according to grade of PanINs, fibrosis, and inflammation in the extracellular matrix as described previously (14).

Fibrosis analysis of pancreas microenvironment

Fibrosis within the pancreatic tissue was evaluated by Masson's trichrome stain. Images ($n = 5$) were taken of each sample from the slides using an Olympus BX61 microscope with a DP73 camera such that a total of $n = 45$ images were taken in the control group and a total of $n = 50$ images were taken of the PAS-treated pancreas. The mean densitometry value per slide was calculated using ImageJ computer software between the mean values for fibrosis of the controls ($n = 9$) to the mean values for fibrosis of the PAS-treated mouse pancreas ($n = 10$).

Evaluation of CCK-B receptors in mouse pancreas tissues

Tissue sections (5 μm) were prepared from the paraffin-embedded mouse pancreas tissues. The tissues were reacted with a goat polyclonal CCK-BR primary antibody (Abcam, cat # ab7707) at 1:200 overnight at 4°C after deparaffination and antigen retrieval procedures. Slides were incubated with biotinylated secondary antibody (R&D Systems cat # CTS008) for 60 minutes, then reacted with DAB Chromogen (R&D Systems cat # CTS008), and counterstained for 1 minute at room temperature with hematoxylin (Fisher, Harris Modified Hematoxylin), blued in 1% ammonium hydroxide and mounted with Permount (Fisher Chemical, cat # UN1249). Images were captured using an Olympus BX61 microscope with a DP73 camera.

CCK-B receptor mRNA expression by real-time qRT-PCR was performed to compare the expression in control versus PAS-treated pancreas tissues. RNA was extracted (Qiagen) from control and PAS-treated mouse pancreata. cDNA was generated and subjected to qRT-PCR using SYBR Green (Life Technologies) in an Applied Biosystems 7300 thermal cycler with the following conditions: initial incubation for 10 minutes at 95°C followed by 40 cycles of 95°C × 30 seconds, 60°C × 1 minutes, and 72°C for 30 seconds with selective CCK-BR primers as follows: 5′GATGGCTGCTACGTGCAACT3′ (forward) and 5′CGCACCACCCGCTTCTTAG3′ (reverse). HPRT was used as a reference gene and HPRT murine primers were as follows: 5′TCAGTCAACGGGGGACATAAA 3′ (forward); and 5′GGGGCTGTACTGCTTAACCAG3′ (reverse).

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Immunohistochemical staining for immune cells

To study the effects of PAS vaccination on cancer promoting M2-polarized macrophages in the pancreas microenvironment, tissue sections (5 μm) were prepared from the paraffin-embedded mouse pancreas tissues. These sections were deparaffinized and hydrated in xylene and descending grades of alcohol. After rinsing in PBS, heat-induced epitope retrieval (HIER) was performed by immersing the tissue sections in Target Retrieval Solution, low pH (DAKO) in the PT Link (DAKO). The endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide for 10 minutes. Another 10 minutes block with 10% normal goat serum was performed to reduce the background, and then the slides were washed in buffer. This procedure was followed by incubation with rabbit polyclonal antibody against arginase-1 (Thermo Fisher Scientific, cat # PA5–29645) at a dilution 1:1,800 for 1 hour at room temperature. Slides were exposed to the appropriate horseradish peroxidase (HRP) labeled polymer for 30 minutes. The antibody reaction was detected using diaminobenzidine (DAB) as chromogen. Sections were counterstained with Hematoxylin (Sigma, Harris Modified Hematoxylin). Negative controls were obtained by omitting the primary antibody in the above procedure on consecutive tissue sections.

Immunohistochemical staining of mouse tissue was performed for total number of macrophages in the pancreas microenvironment on tissue sections (5 μm) using F4/80 antibody (e-Bioscience, cat # 14–4801–85) against mouse, made in Rat at a titer of 1:40 overnight at 4°C. HIER was performed by immersing the tissue sections at 37°C for 14 minutes in pronase. Immunohistochemical staining was performed using a HRP-labeled polymer from Vector MP-7444, according to the manufacturer's instructions. Slides were exposed to the Impress anti-Rat IgG (mouse adsorbed) labeled polymer for 30 minutes and DAB chromogen (Dako) for 5 minutes. Slides were counterstained with Hematoxylin (Sigma, Harris Modified Hematoxylin) at 1:10 dilution for 2 minutes at room temperature, blued in 1% ammonium hydroxide for 1 minute at room temperature, dehydrated, and mounted with Acrymount. Sections with the omitted primary antibody were used as negative controls.

For analysis of $CD8^+$ T lymphocytes in the pancreas microenvironment, tissue sections (5 μm) were treated with 3% hydrogen peroxide and 10% normal goat serum for 10 minutes each, and exposed to primary antibodies for CD8, (1:25, Cell Signaling Technology, cat # 98941) overnight at 4°C. Slides were exposed to the appropriate HRP-labeled polymer for 30 minutes and DAB chromogen (Dako) for 5 minutes. Slides were counterstained with hematoxylin (Sigma), blued in 1% ammonium hydroxide, dehydrated, and mounted with Acrymount.

Tissue sections (5 μm) were reacted with a rabbit monoclonal antibody for Ki67 (Biocare, cat# CRM325; 1:80) to determine the proliferation activity in the pancreas of the control versus PAS-treated mice.

F4/80-, CD8-, and Ki67-stained slides were scanned using an Aperio GT450 machine and images analyzed with software from Aperio Image Scope. The number of total macrophages was analyzed by densitometry with ImageJ software corrected for area of tissue examined. $CD8^+$ stained cells were counted manually and normalized for area of tissue for each pancreas section.

Serum gastrin measurement by ELISA

Blood was collected at the time of euthanasia and serum separated and frozen at −80°C until analyzed for serum gastrin with an ELISA Kit (Enzo Life Sciences, Inc., cat # ADI-900–149). Samples were diluted 1:2 and the assay performed in duplicate.

Western blot analysis

Protein was extracted from mouse pancreas with protein lysis buffer (1 mmol/L EDTA, 0.5% Triton X-100, 5 mmol/L NaF, 6 M urea, 1 mmol/L activated sodium orthovanadate) containing multiple proteinase inhibitors (Thermo Fisher Scientific, cat # A32955). NuPAGE LDS Sample Buffer (Invitrogen, cat # NP0007) was added to each protein lysate at a 4:1 ratio. Samples of equal protein (50 μg) were loaded onto NuPAGE 4% to 12% Bis-Tris gels (Invitrogen, cat # NP0321BOX) and were separated by electrophoresis at voltage 200, then transferred to a nitrocellulose membrane (Thermo Fisher Scientific, cat # 88018) with a voltage 50 for 2 hours. The membrane was blocked in 5% nonfat milk for 1 hour at room temperature, then blotted using the mouse anti-PCNA monoclonal antibody (Proteintech, cat # 60097–1-Ig) at a dilution of 1:5,000 overnight. Then, the blots were incubated with anti-mouse IgG at room temperature for 1 hour, conjugated to HRP, and developed by WesternBright ECL-Spray (Advansta, cat # K-12049-D50). The Spectra Multicolor Broad Range Protein Ladder (Thermo Fisher Scientific, cat # 26634) was used for molecular weight determinations. For loading normalization, the same membrane was blotted with mouse monoclonal anti-β actin antibody (Invitrogen, cat # MA1–140) at a dilution of 1:1,000 overnight, incubated with anti-mouse IgG at room temperature for 1 hour, and developed by ECL. The Western blot bands were quantified using ImageJ software, then statistically analyzed using the Student *t* test method.

Statistical analysis

Differences on pancreas PanIN grade, fibrosis density, M2-polarized macrophages, F4/80 macrophages, $CD8^+$ T cells, and serum gastrin values between control-untreated and PAS-treated mice were determined using GraphPad Prism version 9 statistical analysis programs. Mean values were compared by Student *t* test between the two groups and significance was set at a confidence level of 95% or $P < 0.05$. For gastrin levels between controls and PAS-treated mice with and without cancer and wild-type mouse, an ANOVA two-way comparison was performed between the groups. Differences in the incidence of cancer in each group were analyzed using a Fisher exact test.

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Results

Effects of PAS vaccination on PanINs

Treatment with PAS was started when the mice were 3 months of age, when the pancreas already has established low-grade PanINs (Supplementary data Supplementary Fig. S1). The results of the PanIN grades between the 8-month-old pancreata of each group are shown in **Fig. 1**. High-grade PanIN-3 lesions were significantly reduced in the pancreas of PAS-treated mice compared with controls (**Fig. 1A**; $P = 0.0077$). The number of low-grade PanIN-2 lesions and PanIN-1 lesions are shown between the PAS-treated mice and controls in **Fig 1B** and **C**, respectively. There was no statistical difference in the number of low-grade PanIN lesions between PAS-treated mice and controls. To correct for any variability in tissue size from sectioning, the PanIN grade and number was represented as a percentage of PanIN grade per area of tissue (**Fig. 1D**). Control mice had 55% more total ($n = 22{,}774$) PanIN lesions per area of tissue than the PAS-treated mice ($n = 12{,}425$). When euthanized at 8 months of age, 62.3% of the PanINs in control mice were high-grade PanINs (PanIN-3 lesions, i.e., carcinoma *in situ*) compared with 39.3% in the PAS-treated mice (**Fig. 1D**). Although the numbers were small, 33.3% of control mice developed invasive carcinoma by month 8 and only 1 of the 10 PAS-treated mice developed cancer (**Fig. 1E**). Because of the small numbers, this value did not reach statistical significance ($P = 0.3$).

Serum blood gastrin levels were measured by ELISA. PAS-treated mice had mean gastrin levels of 73 ± 11 pg/mL compared with a mean value of 93 ± 13 pg/mL in the untreated control mice (**Fig. 1F**). This 22% decrease in serum gastrin levels in PAS-treated mice did not reach statistical significance. Gastrin levels are shown for the mice with and without cancer compared with gastrin levels of an age-matched wild-type C57BL/6 mouse (**Fig. 1G**), and those mice with cancer had significantly higher gastrin levels ($P = 0.006$). When we examined the gastrin levels of the mice with cancer, we found that the one PAS-treated mouse with cancer had an increased gastrin level of 126 pg/mL, a value similar to the mean gastrin level (139 ± 14.8 pg/mL) of the 3 control mice that also developed cancer (**Fig. 1G**). This finding would suggest that this particular PAS-treated mouse did not respond to the PAS vaccination.

Representative H&E histologic images of the pancreata from untreated control mice showed high-grade PanINs with complete disruption of the normal pancreatic architecture and extensive fibrosis (**Fig. 2A** and **B**). An image taken from a control mouse with invasive pancreatic cancer is shown in **Fig. 2C**. In contrast, representative images of pancreata from PAS-treated mice demonstrate fewer PanIN-3 lesions and preservation of much of the normal pancreas architecture (**Fig. 2D–F**). The pancreas from PAS-treated mice showed 59% more normal pancreatic acinar cells compared with controls. A representative image taken at a lower magnification of the pancreas from a control mouse shows distortion of the normal pancreas architecture and near complete replacement of the pancreas tissue with PanIN lesions and fibrosis (**Fig. 2G**). A representative image of the pancreas from a PAS-treated mouse at the same magnification exhibits fewer PanINs with some preservation of the normal pancreatic architecture (**Fig. 2H**).

Effects of PAS on pancreatic fibrosis

One of the characteristics of pancreatic cancer is the formation of dense fibrotic tissue (20) surrounding the tumor, rendering the tumor less permeable to chemotherapeutic agents (21) and immune cells (22, 23). Extensive fibrosis was revealed by Masson's trichrome stain in the pancreas extracellular matrix of control mice (**Fig. 3A**), while there was significantly less fibrosis observed in PAS-treated mice (**Fig. 3B**). Quantitation of the fibrosis density by morphometric computerized analysis showed that the amount of pancreatic tissue fibrosis was 50% less in the mice vaccinated with PAS as compared with the pancreas of control mice (**Fig. 3C**), and this difference was significant ($P = 0.0001$).

PAS vaccination decreases protumorigenic M2 macrophages

During pancreatic carcinogenesis, the number of protumorigenic macrophages increases in number in the extracellular matrix of the microenvironment surrounding the PanIN lesions (22, 23). The procarcinogenic macrophages stain arginase positive and polarize as M2 macrophages (24). The arginase positive macrophages were abundant in the pancreas microenvironment of the control mice (**Fig. 4A**), while the pancreas microenvironment of PAS-treated mice had significantly fewer M2 macrophages (**Fig. 4B**). Computer analysis of M2 macrophage number revealed that PAS-treated mice had 4-fold fewer arginase positive macrophages (**Fig. 4C**) than control mice, suggesting that PAS vaccination rendered the pancreas less tumorigenic ($P < 0.001$).

To determine whether PAS therapy decreased the total number of macrophages in the pancreas extracellular matrix, tissues were reacted with an F4/80 antibody and analyzed by densitometry. No statistical difference was found between control and the PAS-treated mice for the immunoreactivity of total macrophages (Supplementary Fig. S2). Representative images from control mouse pancreata reacted with the F4/80 antibody are shown (Supplementary Fig. S2A and S2B). Representative images from a PAS-treated mouse pancreas are shown in Supplementary Fig. S2C and S2D. Densitometry analysis comparing the F4/80 staining macrophages was compared with that of the PAS-treated pancreas F4/80 reacting macrophages (Supplementary Fig. S2E), and no statistical differences were observed between the total F4/80 staining macrophages in these groups. These results suggest that PAS treatment changes the macrophage polarization in the pancreas microenvironment and not the total number of macrophages.

PAS vaccination increases the influx of $CD8^+$ T lymphocytes

The total number of $CD8^+$ cells were evaluated by immunohistochemistry and manually counted. The number of

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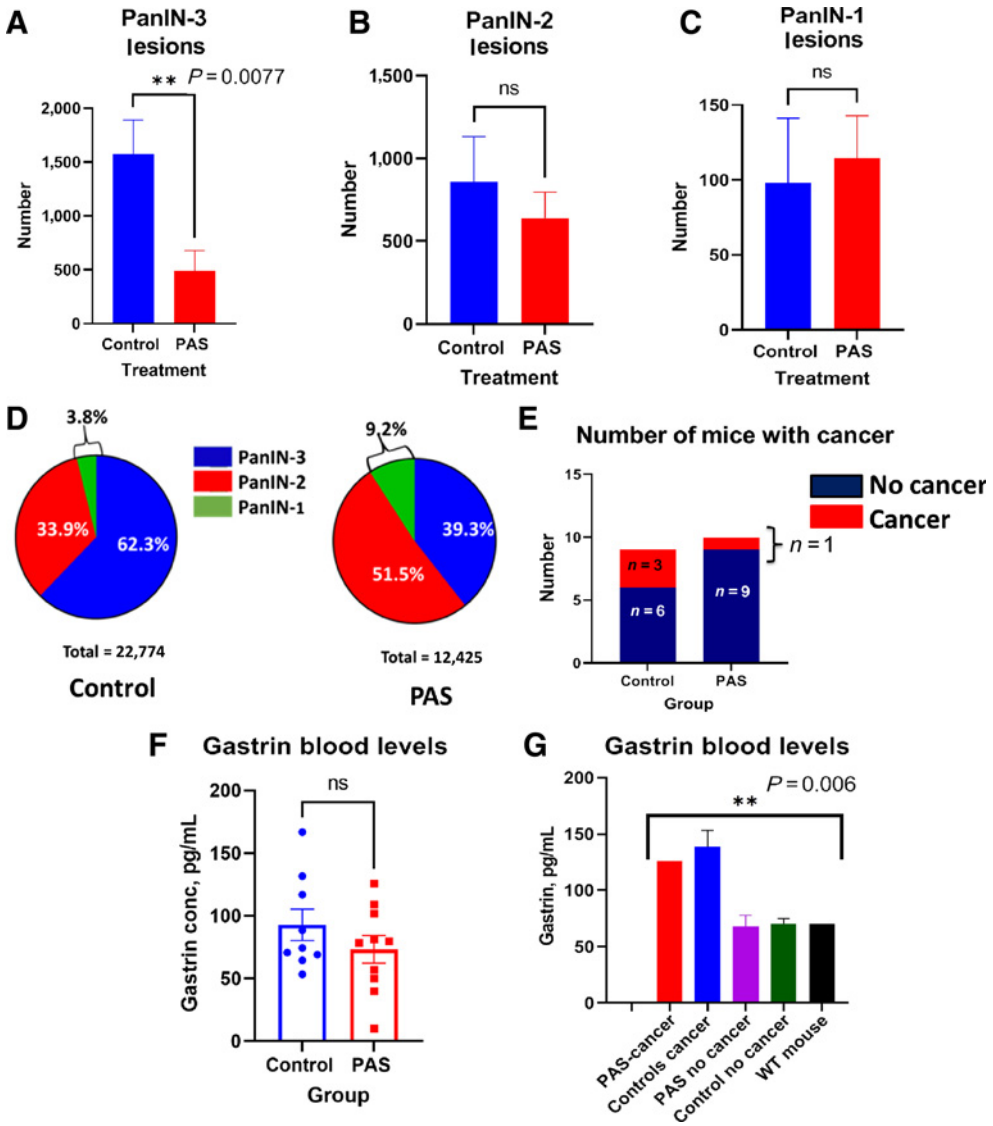

Figure 1.

PAS-treated mice have fewer high-grade PanIN-3 lesions in the pancreas. **A,** The number of PanIN-3 lesions was significantly lower in the pancreas of PAS-treated mice compared with control mice ($P = 0.0077$). **B,** PanIN-2 lesions were 25% less in the pancreas of PAS-treated mice, but this was not significant. **C,** No significant difference was found in the number of PanIN-1 lesions between the controls and PAS-treated mice. **D,** Grade of PanIN lesions is shown as a percentage for each group normalized for the area of the tissue. Control mouse pancreas (left) exhibited a greater percentage of PanIN-3 lesions compared with that of the PAS-treated mouse pancreata (right). **E,** The total number of mice in each group with cancer (red) and without cancer (blue) is shown in each stacked column. Three mice in the control group developed cancer at 8 months while only one in the PAS-treated group developed cancer ($P = 0.3$). **F,** Individual mouse serum gastrin levels in pg/mL are shown in each column. PAS-treated mice had gastrin levels that were 22% lower (73 ± 11 pg/mL vs. 93 ± 13 pg/mL) than control mice but this difference did not reach significance. **G,** Gastrin serum levels are shown according to those with and without cancer and a wild-type C57BL/6 control mouse (black column). Gastrin levels were higher in the mice with cancer compared with the mice without cancer ($P = 0.006$).

$CD8^+$ T cells was significantly increased per area of tissue in mice vaccinated with PAS compared with untreated controls (**Fig. 4D**). A representative image taken from the pancreas of a control mouse reacted with the CD8 antibody, shows few $CD8^+$ T lymphocytes (**Fig. 4E**). In contrast, a representative image taken from the pancreas of a PAS-treated mouse (**Fig. 4F**) showed increased numbers of $CD8^+$ T lymphocytes.

Effects of PAS on cell proliferation

Protein analysis was performed to determine whether PAS decreased PanIN grade by decreasing cellular proliferation or increased apoptosis. Western blot analysis revealed decreased proliferation, as determined by PCNA, in the pancreas tissue of PAS-treated mice compared with controls (**Fig. 5A**). Densitometry of the immunoreactive PCNA band normalized by actin shows the significant difference in the groups consistent

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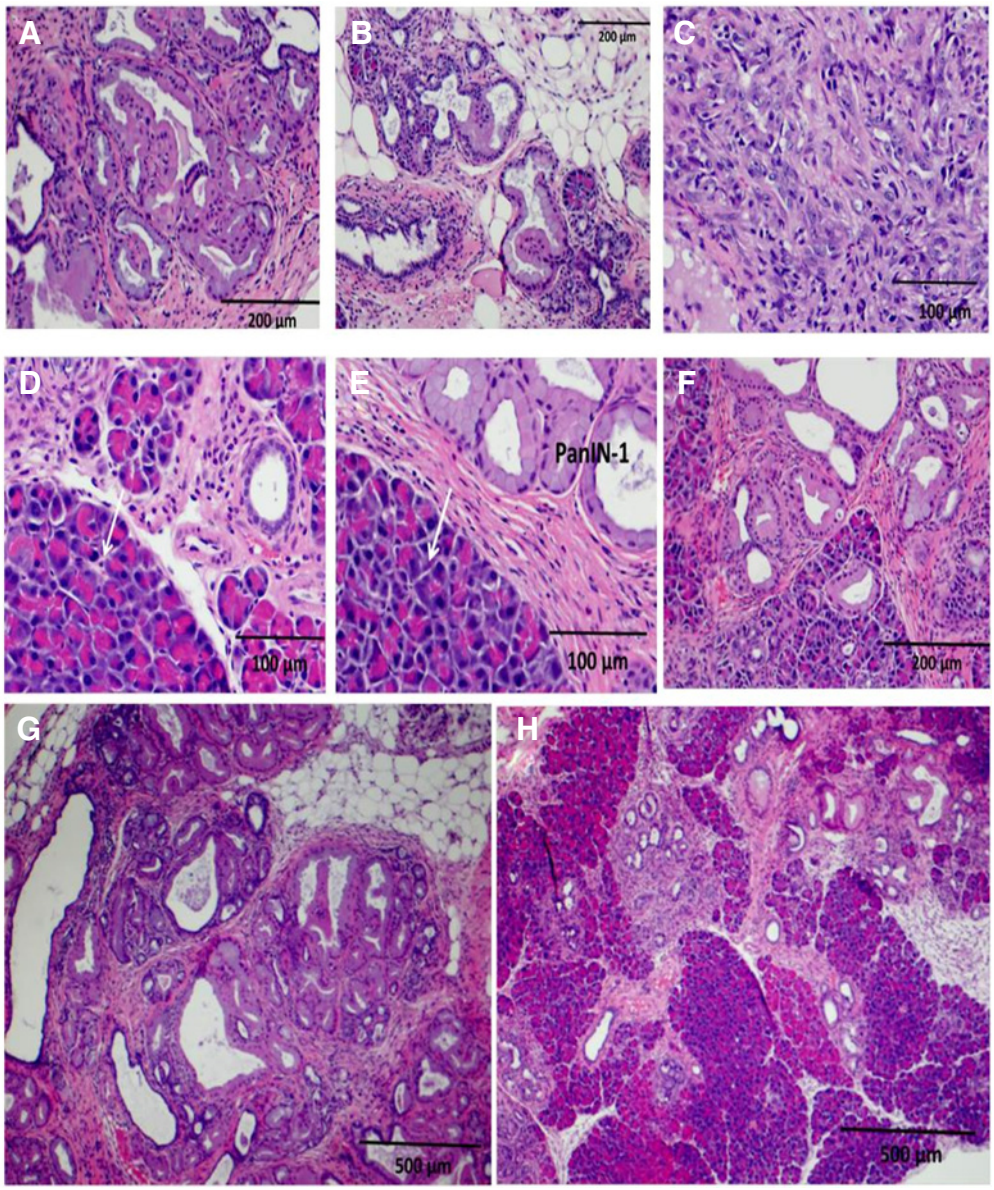

Figure 2.

Hematoxylin and eosin stain of mouse pancreas showing PanIN grade. **A** and **B,** Images of control mouse pancreas with high-grade PanIN-3 lesions and loss of normal pancreatic acinar cells are shown. **C,** Control mouse pancreas with invasive pancreatic cancer is shown. **D–F,** Images from the pancreas from a PAS-treated mouse shows fewer high-grade PanINs with normal pancreatic acinar cells (arrows). **G,** Pancreas from control mouse at low magnification (4×) demonstrates that the pancreas tissue is replaced with extensive PanIN lesions and fibrosis. **H,** Pancreas from a PAS-treated mouse at lower magnification (4×) shows fewer PanINs and preservation of normal pancreas acinar cells.

with decreased proliferation in the pancreas of PAS-treated mice ($P = 0.028$; **Fig. 5B**). Proliferation index was also determined by cellular reactivity to a Ki67 antibody. The number of Ki67-positive cells was numerous in the pancreas of control mice (**Fig. 5C**). Immunoreactivity for Ki67-positive cells was decreased in the pancreas of mice treated with PAS (**Fig. 5D**). Quantitative morphometric analysis of Ki67-positive cells was analyzed and determined to be 5.5-fold less ($P < 0.0001$) in the pancreata of mice treated with PAS (**Fig. 5E**). When the pancreas protein from control mice and PAS-treated mice was compared by ELISA for apoptosis and increased cleaved caspase-3, no statistical difference was observed (**Fig. 5F**). These results indicate that the decreased number of high-grade PanIN-3 lesions in PAS-treated mice was due to a decrease in proliferation rather than by increasing apoptosis.

CCK-B receptor expression

Gastrin mediates its actions through the CCK-B receptor that becomes expressed in PanIN lesions during carcinogenesis (14). Immunoreactivity of the pancreas from the LSL-Kras$^{G12D/+}$;

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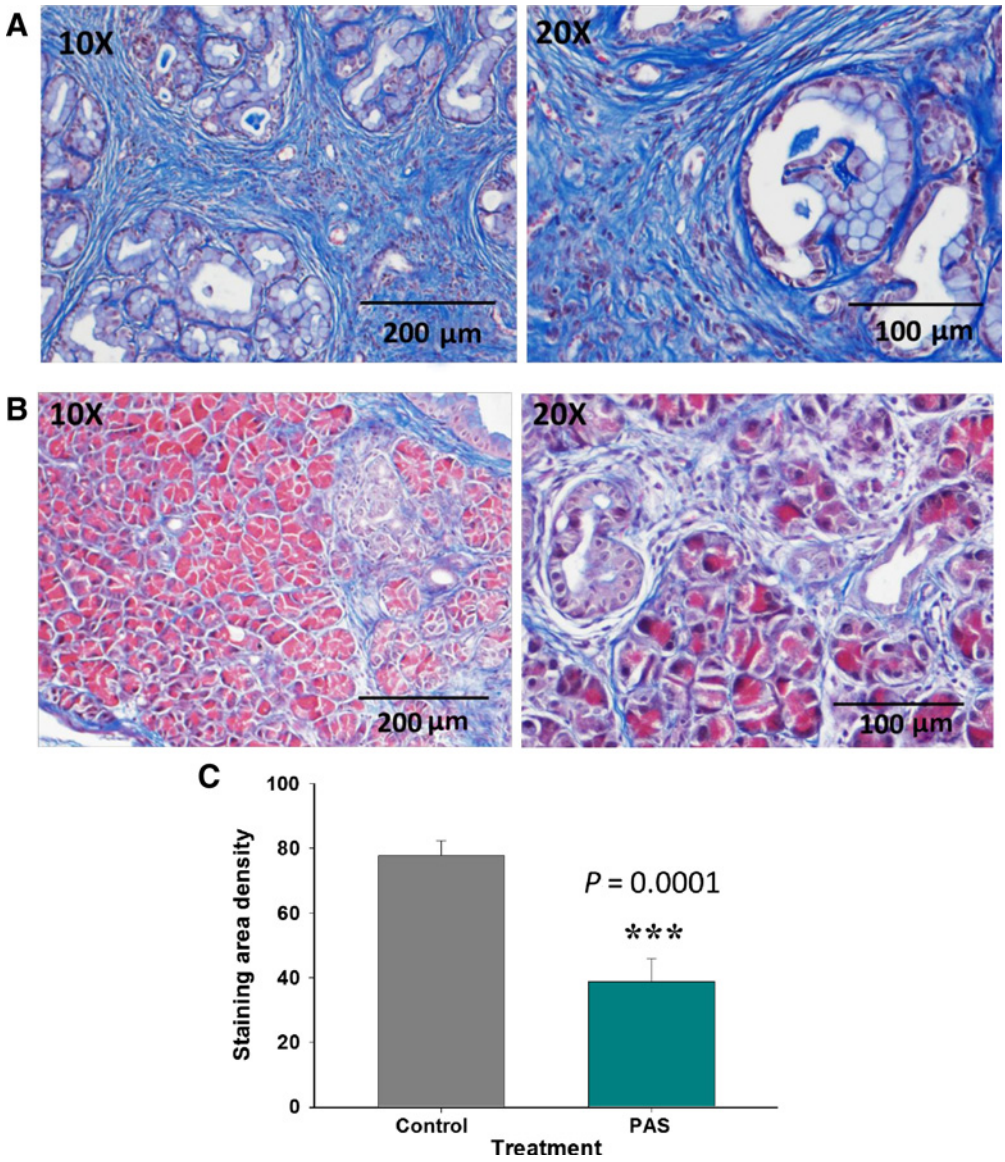

Figure 3.

Pancreatic fibrosis in the pancreas microenvironment by Masson's trichrome stain. **A,** Representative images of pancreas from control KC mice at 8 months of age showing extensive fibrosis (blue stain) at magnifications 10× (left) and 20× (right). **B,** Representative Masson trichrome images of pancreas from age-matched 8-month-old mice vaccinated with PAS show significantly less intrapancreatic fibrosis, magnifications 10× (left) and 20× (right). **C,** Morphometric computerized analysis of fibrosis density shows significantly less fibrosis in the PAS-treated pancreas ($P = 0.0001$).

P48-Cre mice at 8 months of age demonstrate the presence of CCK-B receptor staining in the PanIN epithelial cells (Supplementary Fig. S3A and S3B). Measurement of CCK-B receptor mRNA expression in the pancreas tissues was 5.6-fold lower in PAS-treated mice compared with controls, but this value did not reach statistical significance (Supplementary Fig. S3C).

Discussion

In this investigation, we demonstrated that the progression of precancerous lesions of the pancreas can be prevented by a vaccine that targets gastrin. PAS-treated mice exhibited fewer high-grade PanINs in the KRAS mice pancreas and also a decrease in the incidence of cancer. Proliferation of the pancreas tissue was decreased as demonstrated with less PCNA and fewer total number of PanIN lesions. In part, this antiproliferative effect is mediated by the activation of B cells that produce neutralizing antibodies to gastrin, a known trophic peptide (25), in response to the vaccination. PAS vaccination has also been shown to elicit at T-cell response and activation of memory T lymphocytes that respond when exposed to gastrin with the release of proteases such as granzyme and perforin and cytokines including IFNγ and TNFα (17). Because CCK-B receptors become expressed in early PanIN lesions (14) and

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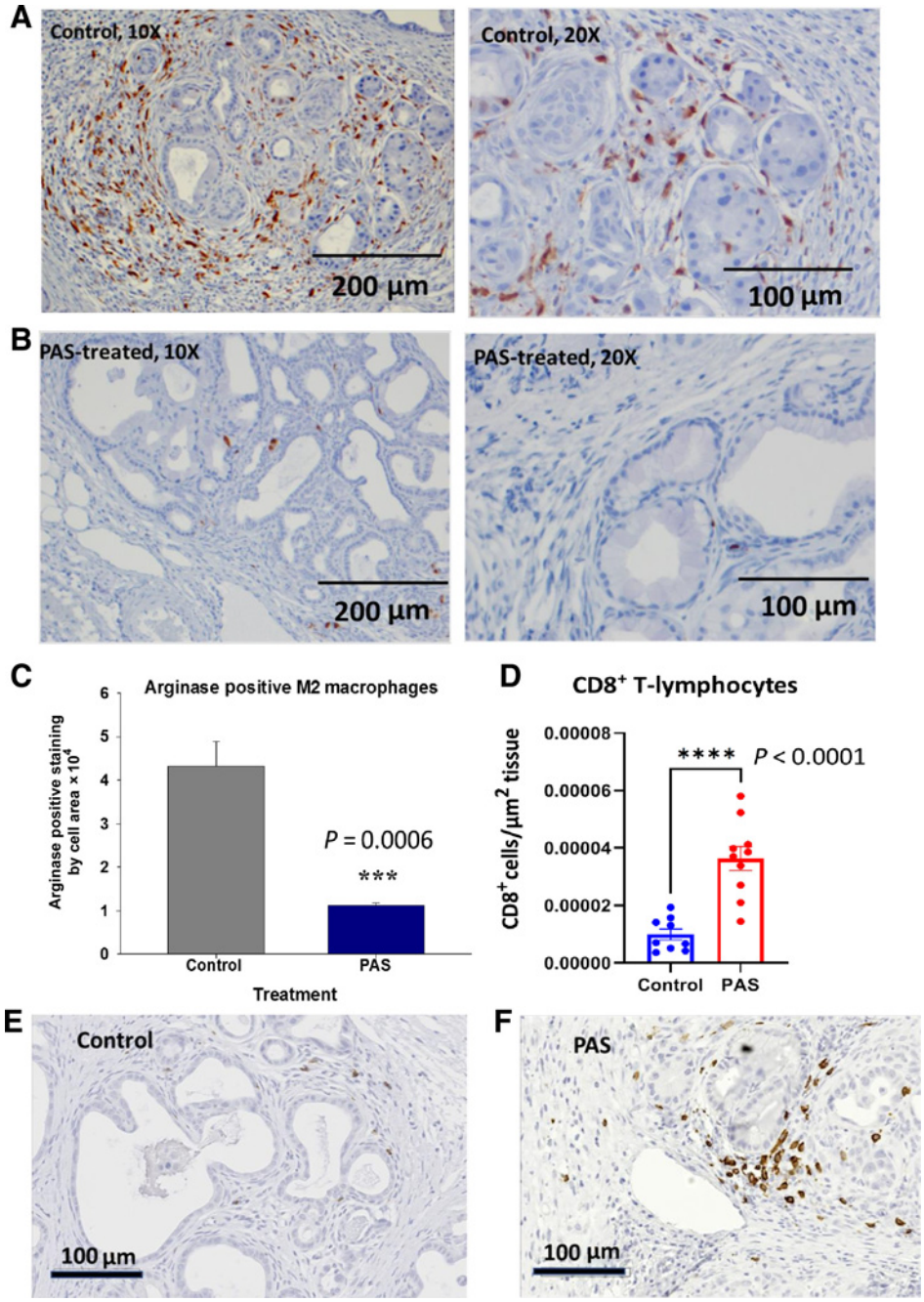

Figure 4.

PAS treatment changes the immune cell signature in the pancreas microenvironment. **A,** Section from representative control mouse pancreas shows numerous arginase positive M2 polarized macrophages (10×, left). Photo of a control mouse pancreas (20×; right) shows arginase positive macrophages surrounding PanIN lesions. **B,** Photo from a pancreas from a PAS-treated mouse shows few arginase positive macrophages (10×; left). Higher magnification (20×) of a pancreas from a PAS-treated mouse shows decreased M2 arginase positive macrophages compared with control mice. **C,** Computer analysis of arginase positive M2 macrophages shows a 4-fold decrease in tumor associated macrophages in the pancreas of PAS-treated mice ($P = 0.0006$). **D,** $CD8^+$ T lymphocytes per area of tissue are shown for each individual sample analyzed. PAS-treated mice exhibited higher numbers of $CD8^+$ immunoreactive T cells in the pancreas compared with controls ($P < 0.0001$). **E,** A representative photo from a control mouse pancreas shows few $CD8^+$ cells in the pancreas extracellular matrix. **F,** Image taken from the pancreas of a PAS-treated mouse shows increased number of $CD8^+$ cells.

gastrin activation of this receptor induces downstream signaling with epithelial cell proliferation (26), interruption of gastrin's actions at the receptor interface most likely resulted in the PanIN arrest.

Another important finding of this investigation involves the alteration of the pancreas microenvironment in PAS-treated mice. During pancreatic carcinogenesis, the pancreatic stellate cells become activated myofibroblasts and deposit a collagenous desmoplasia in the pancreas (20). Stellate cells also have CCK-B receptors (27) and inhibiting gastrin activation of these receptors resulted in less fibrosis in the microenvironment. The fibroblasts of the pancreatic microenvironment communicate with the epithelial cells and immune cells resulting in the activation of cytokines (28). This immune activation leads to the destruction of normal pancreatic tissue and replacement with the precancerous PanIN lesions. Indeed, large sections of the normal pancreas and acinar cells were protected in the PAS-treated mice. Previously, we had shown that intratumoral fibrosis was reduced in mice bearing established pancreatic tumors treated with a combination of PAS and a PD-1 antibody but that monotherapy with PAS or PD-1

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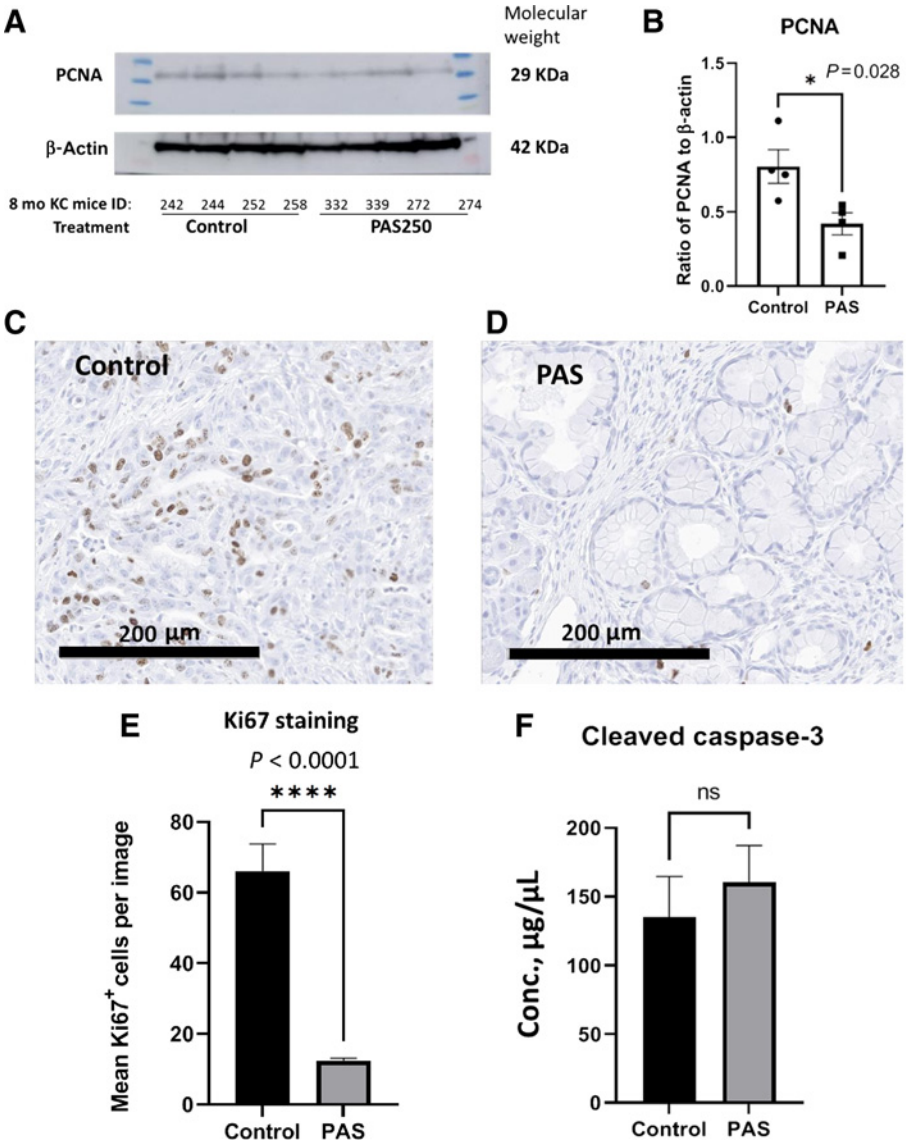

Figure 5.

PAS therapy decreases proliferation of the mouse KRAS pancreas. **A,** Western blot of protein extracts from mouse pancreas reacted with the antibody to the PCNA is shown at the expected 29 kDa size. The protein is normalized to actin with the band shown at the expected 42 kDa size. Mouse identification numbers are shown below the blot and protein ladder size exhibited on each side of the samples electrophoresed. **B,** The Western blot was analyzed by densitometry and the individual samples (dots and squares) and mean ± SEM values (columns, error bars) show a significant decrease in PCNA immunoreactivity in tissue from PAS-treated mice ($P = 0.028$). **C,** Representative image from the pancreas of a control mouse reacted with a Ki67 antibody shows numerous immunoreactive proliferating cells. **D,** A representative image is shown from the pancreas of a PAS-treated mouse with Ki67 staining. **E,** Quantitative analysis of Ki67 immunoreactive cells is shown. Columns represent the mean number of $Ki67^+$ cells counted per high power (200 μm) image with $n = 10$ images per slide. The number of $Ki67^+$ proliferating cells is significantly increased in the pancreas tissue of control mice compared with the pancreas tissue from PAS-treated mice ($P < 0.0001$). **F,** Columns represent the mean ± SEM of pancreatic protein cleaved caspase-3 levels in μg/μL as measured by ELISA revealed no significant difference between control and PAS-treated mice.

antibody failed to reduce fibrosis (17, 18). One possible explanation for the marked decrease of fibrosis in the current investigation with PAS monotherapy could be that in the current investigation, PAS was administered over a longer duration in the KRAS mice compared with the tumor-bearing mice of the previous study (months vs. weeks) and several boosters were administered in the current investigation.

This investigation demonstrated that PAS therapy changes the immune cell signature of the pancreas extracellular matrix, rendering it less carcinogenic. An important immune cell that promotes pancreatic carcinogenesis is the M2 macrophage (22, 23). The untreated control mouse pancreas microenvironment becomes infiltrated with abundant M2 arginase positive macrophages during carcinogenesis. PAS vaccination decreases the number of M2-polarized macrophages without decreasing the total number of tissue associated macrophages that are important for immune surveillance. This change in polarization of macrophages further contributes to decreasing the carcinogenic potential of the pancreas.

One reason pancreatic cancer has not responded to immune checkpoint antibody therapy (29) is related to the lack of $CD8^+$ T lymphocytes in the pancreas tumor microenvironment (30). Improved patient survival with pancreatic cancer has been directly correlated to the number of $CD8^+$ T cells in the pancreas microenvironment (31). PAS therapy significantly increased the number of $CD8^+$ immunoreactive T cells in the pancreas microenvironment in the KRAS-mutant mouse model. In a prior study in mice bearing pancreatic tumors, PAS therapy increased intratumoral $CD8^+$ T cells while also decreasing the immunosuppressive T regulatory cells (17).

PAS is a peptide vaccine that selectively targets gastrin, a known trophic peptide that stimulates pancreatic cancer in both a paracrine (32) and autocrine (12) fashion. Gastrin and

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its related peptide, cholecystokinin (CCK), both mediate their proliferative effects through activation of the CCK-BR with equal affinity (33); however, only gastrin can stimulate growth in an autocrine fashion. Although CCK peptide expression may be found in pancreatic cancer cells, CCK does not stimulate growth in an autocrine fashion. Neither treatment of pancreatic cancer cells with selective CCK antibodies nor the downregulation of CCK mRNA and peptide by shRNAs alters growth *in vitro* or *in vivo* (34). Conversely, when gastrin mRNA expression was downregulated, the same pancreatic cancer cells failed to produce tumors in spite of having sustained levels of endogenous CCK (34). CCK blood levels may be increased by consumption of a diet high in saturated fats, which in turn can stimulate pancreatic cancer growth in an exogenous fashion through the CCK receptor (35, 36). Epidemiologic studies have shown that the incidence of pancreatic cancer is increased in countries that consume high fat diets, especially saturated fat (37–39) and long-chain saturated fatty acids are effective releasers of CCK (40). Obesity has been associated with an increased risk for pancreatic cancer (41, 42) and upregulation of CCK in pancreatic islet cells has been associated with obesity in the Leptin$^{ob/ob}$ mice mouse models (43, 44). Because both CCK and gastrin stimulate pancreatic carcinogenesis through the CCK-BR, the increased expression of CCK-BR in pancreatic carcinogenesis seems to be an important driver. Our prior work has demonstrated that diets high in saturated fat increase the expression of the CCK-BR by downregulating miR-148a levels (45). And during pancreatic carcinogenesis in the mutant *KRAS* mouse models, levels of miR-148a decrease (46) as CCK-BR expression increases (16). Although PAS vaccination does not alter CCK levels, PAS therapy will neutralize endogenous gastrin levels and decrease signaling of the CCK-BR. In our *KRAS* model, PAS vaccination slowed PanIN progression but did not completely arrest PanIN progression perhaps because CCK levels were unaltered in our model. Conceivably, combination therapy with PAS and a CCK-receptor antagonist, such as proglumide (47), would provide full protection for PanIN progression to pancreatic cancer.

Currently, there are no therapies to prevent pancreatic cancer. Morrison and colleagues in their review on Immunotherapy and Prevention of Pancreatic Cancer commented that "Elimination of the precursor lesion might be enough to prevent the development of malignancy or at least 'reset the clock' (10)." Indeed, our findings support that immunization with PAS definitively decreases the progression low grade PanIN precursor lesions to high-grade PanIN-3 lesions in this LSL-Kras$^{G12D/+}$; *P48-Cre* mouse model that is destined to develop pancreatic cancer. Although we waited until 3 months of age to initiate therapy after establishment of PanIN lesions, one could speculate that administration of therapy with PAS at an earlier time point would be more protective. Populations that could benefit immediately from our research include those that are considered high risk for pancreatic cancer (5, 10), such as those with a family history of pancreatic cancer, chronic pancreatitis, or new onset diabetes. Those with BRCA2 germline alterations or hereditary pancreatitis may also benefit from PAS vaccination. Currently, these populations with high risks or family history of pancreatic cancer undergo MRI imaging surveillance and occasionally endoscopic ultrasound, but these techniques are only for surveillance and are not preventive. PAS vaccination could possibly offer a novel approach to prevent pancreatic cancer in these high-risk populations. Another strategy for the clinical use of PAS could include vaccinating those that had a surgical resection/Whipple procedure for pancreatic cancer to prevent tumor recurrence. Although curative resection is attempted in up to 20% of those with pancreatic cancer, the 5-year survival for this group is still only 20% to 30% at best due to recurrence of microscopic disease. Treatment with PAS may help decrease recurrence after surgery. Because PAS has already been safely tested in human subjects with pancreatic cancer, strategies to develop PAS as a cancer vaccination in high-risk subjects to prevent cancer (rather than just monitoring them) may improve survival from this devastating disease.

Authors' Disclosures

J.P. Smith reports grants from Georgetown University during the conduct of the study; other support from Cancer Advances outside the submitted work as well as a patent pending. L. Sutton reports a patent for 17/148,159 pending and is the president of Cancer Advances. A. Cato reports a patent for 17/148,159 pending and is the CEO of Cancer Advances. No disclosures were reported by the other authors.

Authors' Contributions

J.P. Smith: Conceptualization, resources, data curation, supervision, funding acquisition, investigation, methodology, writing–original draft, project administration, writing–review and editing. **H. Cao:** Data curation, formal analysis, investigation, methodology, writing–review and editing. **W. Chen:** Data curation, writing–review and editing. **B. Kallakury:** Data curation, formal analysis, writing–review and editing. **T. Phillips:** Conceptualization, funding acquisition, methodology, writing–review and editing. **L. Sutton:** Conceptualization, funding acquisition, writing–review and editing. **A. Cato:** Conceptualization, funding acquisition, writing–review and editing.

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