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ORIGINAL ARTICLE

Gastrin vaccine improves response to immune checkpoint antibody in murine pancreatic cancer by altering the tumor microenvironment

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Abstract

Pancreatic cancer has been termed a 'recalcitrant cancer' due to its relative resistance to chemotherapy and immunotherapy. This resistance is thought to be due in part to the dense fibrotic tumor microenvironment and lack of tumor infiltrating CD8 + T cells. The gastrointestinal peptide, gastrin, has been shown to stimulate growth of pancreatic cancer by both a paracrine and autocrine mechanism. Interruption of gastrin at the CCK receptor may reduce tumor-associated fibrosis and alter tumor immune cells. Polyclonal Ab Stimulator (PAS) is a vaccine that targets gastrin and has been shown to prolong survival of patients with pancreatic cancer. Here, we report that PAS vaccination monotherapy elicits both a humoral and cellular immune response when used in immune competent mice-bearing pancreatic tumors and that PAS monotherapy produced a marked T-cell activation and influx of CD8 + lymphocytes into pancreatic tumors. Isolated peripheral lymphocytes elicited cytokine release upon re-stimulation with gastrin in vitro demonstrating specificity of immune activation for the target peptide. Combination therapy with PAS and PD-1 Ab activated CD4 −/CD8 − TEMRA cells important in T-cell-mediated tumor death and memory. Tumors of mice treated with PAS (250 µg) or PAS (100 and 250 µg) in combination with a PD-1 Ab were significantly smaller compared to tumors from PBS or PD-1 Ab-treated mice. When PAS was given in combination with PD-1 Ab, tumors had less fibrosis, fewer inhibitory Treg lymphocytes, and fewer tumor-associated macrophages. These findings reveal a novel approach to improve treatment strategies for pancreatic cancer.

Keywords Tumor microenvironment · Vaccine · Gamma–delta T cells · NKT cells · Cellular immunity · Gastrin

Abbreviations

Ab	Antibody
CCK	Cholecystokinin
GI	Gastrointestinal
i.p.	Intraperitoneal
mT3	mT3-2D
PAS	Polyclonal antibody stimulator
PAS100	100 µg Polyclonal antibody stimulator
PAS250	250 µg Polyclonal antibody stimulator
PDAC	Pancreatic ductal adenocarcinoma
s.c.	Subcutaneous
TAMs	Tumor-associated macrophages
TEMRA	Terminally differentiated T cells
TME	Tumor microenvironment

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Introduction

In spite of success in treatment of other cancers, no improvement has occurred in survival of patients with pancreatic ductal adenocarcinoma (PDAC), [1, 2] which carries the poorest prognosis of all gastrointestinal (GI) malignancies [3]. Currently, the 5-year survival rate for PDAC is about 8%, the lowest of any cancer [4]. Reasons for poor survival rates include inability to diagnose this disease in the early stages, its dense fibrotic

microenvironment [5], and its resistance to both chemotherapy and immunotherapy [6]. The host immune response is another key factor contributing to the recalcitrant and aggressive nature of PDAC. Immune cells that are most prominent in the microenvironment of PDAC do not support anti-tumor immunity [7]. Instead, inflammatory cell components (which include macrophages, Tregs, and neutrophils) promote tumor growth and invasion. PDAC characteristically employs many tools to escape or defeat anti-tumor immune responses and is supported by components of tumor metabolism that regulate these responses [8, 9]. PDAC is considered a non-immunogenic cancer, because its tumor microenvironment (TME) has a predominance of immune suppressing Tregs, an absence of tumor infiltrating effector (CD8 +) T cells [7, 8, 10], and it does not respond clinically to PD-1 antibodies (Abs) [11]. Concurrently, the discovery of the immune checkpoint pathway blockade has resulted in an upsurge in the number of new cancer therapeutics that has revolutionized the management of patients diagnosed with recalcitrant malignancies [12]. Unfortunately, immune checkpoint antibodies to date have not been successful in the treatment of pancreatic cancer [11, 13–16]. Investigators have been searching for companion agents to combine with immune checkpoint antibodies to increase their efficacy. Strategies to modify the immune phenotype of the pancreatic cancer microenvironment and thereby make it more responsive to immune therapies and immune checkpoint blockade are active areas of investigation [17], and the focus of this body of research.

Many advances in cancer therapy have come from identification and blockade of tumor-specific receptors or growth factors [18]. One such growth factor is the gastrointestinal peptide, gastrin, which has been shown to stimulate growth of pancreatic cancer [19] when exogenously applied to pancreatic cancer cells in vitro. Gastrin is also secreted by pancreatic cancer cells, and thus stimulates their growth through an autocrine mechanism [19, 20]. When expression of the gastrin gene is down regulated by RNA interference techniques, pancreatic tumors fail to grow and metastases are prevented [21]. Gastrin mediates its actions through cholecystokinin (CCK) receptors that are expressed on pancreatic cancer cells [22, 23], immune cells [24], and pancreatic stellate cells/fibroblasts [25, 26]. CCK receptor blockade has been shown to decrease pancreatic tumor fibrosis [27] and alter the immune cell signature of the TME [24, 28].

We studied a tumor-associated, antigen-based vaccine, exploiting involvement of gastrin as a key trophic factor for pancreatic cancer and a regulator of the pancreas TME. The vaccine 'PAS' elicits its effects through an active humoral immunity against gastrin-17. PAS (formerly called G17DT or Gastrimmune) is 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 stimulates the body to produce specific, and high-affinity polyclonal anti-gastrin antibodies.

Preclinical studies using PAS were performed in animal models with gastrointestinal cancers (colon [29], gastric [30], and pancreatic cancer [31]) and showed that tumor growth was reduced in animals that mounted anti-gastrin antibodies following vaccination with PAS. To date, six pancreatic cancer clinical trials with more than 450 pancreatic cancer patients have been completed; four examined the use of PAS as a monotherapy and two examined the use of PAS in combination with gemcitabine. The majority of subjects vaccinated with PAS developed neutralizing antibodies to gastrin and in one study at least 25% survived for 305 days (Study PC-6) [32]. Overall, PAS-treated patients with confirmed Ab titers to gastrin had a median survival of 190 days compared to 84 days for the placebo group. Since some long-term survivors (alive after week 104) were identified in the clinical studies, we hypothesized that PAS therapy may have also elicited a T-cell memory response in addition to the known anti-gastrin neutralizing antibodies.

The current investigation was undertaken to determine to what extent vaccination with PAS in immune competent mice mounts a gastrin-dependent T-cell response. In addition, this study examined the role of combined immune therapy using PAS together with a PD-1 immune checkpoint Ab on the growth of pancreatic cancer and the TME in a syngeneic murine model of pancreatic cancer.

Materials and methods

Cells and animals

The murine pancreatic cancer cell line used in this investigation, mT3-2D (mT3), was developed from organoids isolated from mutant *Kras*$^{+/LSL-G12D}$; *Pdx1-Cre* mouse PDAC lesions [33]. We previously characterized this cell line and found that it produces gastrin and has CCK-B receptors [34]. The cell line is also syngeneic to C57BL/6 mice, facilitating the ability to study tumor response to a vaccine in immune competent mice. Mice were housed in the Comparative Medicine facility at Georgetown University with 5 mice per cage in filter top cages and fed standard chow and water ad libitum. The rooms were on automatic lighting with a 12-h on–off cycle.

Study design and treatments

Sixty male (6 weeks) C57BL/6 mice were injected subcutaneously into the right flank with 500,000 mT3 murine pancreatic cancer cells. On the sixth day after inoculation 100% of the mice had a palpable tumor and were allocated

into one of six groups of $N = 10$ mice each so that the baseline tumor volume was equal in all groups. Groups consisted of mice treated with: PBS (control), 100 µg PAS (PAS100), 250 µg PAS (PAS250), PD-1 Ab (150 µg), and combination therapy with PD-1 Ab (150 µg) plus 100 µg PAS or PD-1 Ab (150 µg) plus 250 µg PAS administered by intraperitoneal (i.p.) in a volume of 100 µL. PAS was administered at the time of randomization (baseline time = week 0) and again at week 1, and week 3. PD-1 Ab (Bio X cell; cat# BE0146; Clone: RMP1-14, West Lebanon, NH) was given five times during the study (baseline, days 4, 8, 15 and 21). Control mice received PBS (100 µL) on the same days that PAS was administered. Tumor volumes were measured weekly [day 7 (baseline), day 14, day 21, and day 28] using calipers and were calculated as length × (width)^2 × 0.5 starting at baseline (1 week after tumor cell inoculation); tumor volumes were re-measured also at euthanasia on day 31. A diagram of the study design is shown in Fig. 1. The experiment was repeated to confirm the results. In the second experiment, all the treatments were administered i.p. injection with the exception of the PAS250 dose. The 250 µg dosage of PAS was administered subcutaneously in the repeat experiment rather than i.p. at the recommendation of the staff veterinarians, since three mice injected i.p. with 250 µg PAS in the first experiment developed peritonitis.

Spleen T-cell isolation

At the termination of the experiments, the spleen was removed from each animal and placed in a 60 mm dish containing 5 ml RPMI1640 medium. Spleens were mechanically disrupted using a razor blade, and medium containing the spleen tissue suspension was filtered through a 100 µm cell strainer into a 50 ml tube. Material in the strainer was rinsed with medium until final volume of the filtrate was 40 ml. Spleen filtrate was filtered again using a 40 µm cell strainer and material collected in another 50 ml tube. The tube was centrifuged at 1500 rpm for 5 min at 4 °C to pellet the cells. Supernatant was removed and the cell pellet resuspended in 40 ml PBS. Cells were re-pelleted by centrifugation at 1500 rpm for 5 min at 4 °C. The supernatant was discarded, the cell pellet was resuspended in 3 ml washing buffer (PBS with 2 mM EDTA and 0.5% bovine serum albumin), and then slowly overlaid onto 5 ml of 1.084 Ficoll medium (Sigma-Aldrich) in a 15 ml tube. After centrifugation at 2100 rpm for 20 min with deceleration set to zero, the lymphocytes were collected from the white layer between buffer and the Ficoll. Lymphocytes were washed two additional times, resuspended in medium, and counted.

Flow cytometry

Flow cytometry was performed to measure surface Ab staining of spleen mononuclear cells. One million viable

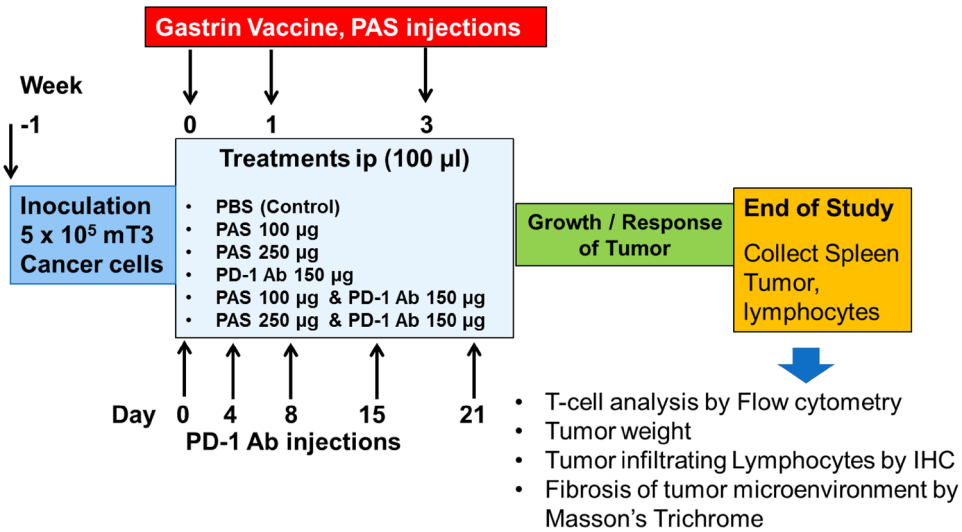


Fig. 1 Experimental study design and treatments. Sixty male C57BL/6 mice were inoculated subcutaneously with 500,000 mT3 murine pancreatic cancer cells. After 1 week, 100% of the mice had palpable tumors and they were randomized to one of 6 treatment groups ($N = 10$) with equal tumor size on Day 0. Mice received PBS (control), PD-1 Ab (150 µg), PAS (100 or 250 µg), or combined therapy with PAS (100 µg or PAS 250 µg) and PD-1 Ab (150 µg). PAS was given 3 times at week 0, 1, and 3. PD-1 Ab and PBS (control) were given 5 times on days 0, 4, 8, 15, and 21. All treatments were given in a volume of 100 µL by the intraperitoneal route. In the second experiment, all injections were also administered by the i.p. route except the PAS250 was given s.c. Tumors volumes were measured weekly. The experiment was repeated once

lymphocytes were added to 5 ml clear tubes (cat# 352054; BD Falcon, Bedford, MA), volumes were equalized with PBS, and cells were pelleted by centrifugation at 1500 rpm for 5 min. After washing with PBS, and re-pelleting, 50 µl of pre-diluted Zombie NIR™ brand fixable viability solution (Biolegened®, San Diego, CA) was added to the cells, which were then incubated at room temperature in the dark for 20 min. Cells were washed and then blocked by adding 5 µl Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block™ brand reagent; BD Biosciences, San Jose, CA) and incubated for 20 min. T-cell antibodies listed in Supplementary Table 1 [including CD4, CD3, CD62L, CD8a, CD25, CD69, CD44, CD45, NK1.1, TCR γδ, and CD279 (PD-1)] were reacted to the lymphocytes and flow cytometry performed using a FACSARIA™ IIu brand cell sorter (BD Biosciences) with 375 nm, 405 nm, 488 nm and 633 nm laser lines.

Re-stimulation assay

One million washed and viable lymphocytes were added to each well of duplicate 6-well plates in a volume of 2 ml. Brefeldin A solution (BIOLEGEND®, 1000X, cat# 420601) was added at 1 µl/ml to each well. Gastrin-14 peptide (Sigma-Aldrich cat# SCP0152) with the amino acid sequence pEGPWLEEEEEAYGW was added to each well of one plate in a volume of 1 µl/ml for a final gastrin concentration of 1 nM. The duplicate plate was not treated with gastrin-14 and served as a control. The 6-well plates were placed in the cell culture incubator at 37 °C for 6 h. The cells were then removed, washed, and stained with four surface antibodies [CD45 (BV 650), CD3 (BV 510), CD4 (BV 605) and CD8 (BV421)]. The lymphocytes were then washed with PBS and fixed with IC Fixation Buffer (Invitrogen, cat# 00-8222-49) for 20 min at room temperature in the dark. The lymphocytes were then washed and resuspended with 1X Permeabilization buffer (Invitrogen, cat# 00-8333-56), stained with one of the four cytokine antibodies listed in Supplementary Table 2 (including interferon-γ, granzyme-B, perforin, and tumor necrosis factor-α) and incubated at 4° C overnight. The next morning, the cells were washed again with the 1X permeabilization buffer, and subjected to flow cytometry. The re-stimulation test was performed twice ($N=6$ spleens/group).

Acquisition of flow cytometry data was achieved using FCSExpress-6 software (De Novo Software, Glendale, CA), and analysis of data performed using the FlowJo flow cytometry analysis platform (FlowJo LLC, Ashland, OR).

Tumor histology and immunohistochemistry

After 31 days of growth, tumor volumes in the PD-1 Ab group had reached the maximum allowed size according to the IACUC approved protocol. Mice were ethically euthanized by CO_2 asphyxiation and cervical dislocation and were weighed. Pancreatic tumors were excised, weighed, and fixed in 4% paraffin formaldehyde. Eight µm cuts of paraffin-embedded tumors were mounted on slides for staining. Tumor-associated fibrosis was revealed with Masson's trichrome stain. Images from each slide were captured using a 20 × objective lens on an Olympus BX61 microscope with a DP73 camera ($N=10$ per group). Analysis of Masson's trichrome was done using software by Image-J by two investigators blinded to treatment of the area of fibrosis.

For immunohistochemistry analysis of TILs, 5 µm fixed tumor sections were stained with either CD8 Ab (1:75; eBioscience); or Foxp3 Ab (1:30; eBioscience) and immunoreactive cells were counted manually. Tumor-associated macrophages (TAMs) were reacted with an F4/80 Ab (1:40; eBioscience) and images were taken on an Olympus BX61 microscope with a DP73 camera. The number of immunoreactive cells per slide was counted with image-J computer software by investigators blinded to the treatments.

Measurement of PAS-induced anti-gastrin antibodies

Blood was collected at the time of euthanasia and serum analyzed for gastrin specific antibodies with an ELISA. Gastrin peptide in the form of gastrin-17-BSA conjugate was immobilized on Nunc CovaLink plates (ThermoFisher, cat# 478042) according to the manufacturer's instructions. Standard, reference serum consisted of serum pooled from 10 mice immunized with PAS vaccine that was characterized previously as containing anti-gastrin antibodies. The gastrin-17-BSA conjugate target antigen and the reference standard were gifts from Dr. Peter Blackburn (Mercia Pharma Inc., New York, NY). Serum from experimental mice in all six treatment groups were diluted 1:100 in PBS containing 1% BSA and 0.5% Tween 20, and was incubated on gastrin plates for 2 h at room temperature. Plates were washed with washing buffer (PBS containing 0.5% Tween20), and then reacted for 45 min at room temperature with 1:25,000 dilution of secondary Ab consisting of goat anti-mouse HRP conjugate (Pierce, cat#31430). Plates were washed with washing buffer six times, and then incubated with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution at room temperature for 30 min. Levels of HRP reactivity were determined by analyzing optical density at 450 nm on a Perkin Elmer Wallac 1420 Victor2 Microplate Reader.

Statistics

Sample size was based upon the known growth rate of mT3 tumors from our experience in other experiments [28]; it was estimated that $N=8$ mice per group were required to

reach a power of 0.80. However, since approximately 10% of the mice may not have tumor take or 10% may succumb prematurely, we included $N = 10$ per group to consider for the potential 'drop-outs'. Statistical analysis was performed by comparing features of the control group, unless specifically indicated, with those of each experimental group using the ANOVA and student's t test to compare mean values with software from PRISM and Minitab. Mean values were calculated for each group using a statistical program from Minitab, Inc. A p value of < 0.05 is considered statistically significant. A Bonferroni correction was performed when multiple comparisons were made to the control.

Results

PAS vaccination activates T-lymphocytes

T-lymphocytes were isolated from spleen mononuclear cells from mice that had been treated with PBS, PD-1 Ab, PAS100, PAS250, and the combination of PAS100 or PAS250 with PD-1 Ab. The percentage of CD3+, CD4+, and CD8+ T-cell populations in the total T-cell sample isolated from spleen mononuclear cells is shown in Fig. 2a-1. This figure shows that PD-1 Ab, PAS250, and PAS250/PD-1 Ab-treated mice have a lower percentage of CD3+ T cells in viable CD45+ spleen PBMC. Mice treated with PAS100 or PAS100/PD-1 Ab had significantly fewer total CD4 + and CD8 + cells compared to PBS-treated mice. The percentage of CD3+ CD4–CD8 (double negative)–T cells for each individual mouse in the six treatment groups is represented in Fig. 2a-2. CD3 + CD4–CD8–T cells are markedly increased in percentage relative to that of PBS controls in mice treated with both PAS100 ($p < 0.005$) and PAS250 ($p < 0.05$) alone or in combination with PD-1 Ab. Representative flow cytometry images of viable T cells (CD3 +), separated by CD4 and CD8, from spleen mononuclear cells of mice treated with PBS, PD-1 Ab, PAS100, PAS250, PAS100/PD-1 Ab, and PAS250/PD-1 Ab are shown in Fig. 2b.

PAS vaccination elicits an increase in terminally differentiated memory T cells

To define more clearly the subpopulation of T cells within CD3 +/CD4–CD8 lymphocytes, further T-cell lymphocyte antigen flow cytometry experiments were performed and cells were separated by CD44 and CD62L expression. In particular, a T-cell subpopulation was initially isolated that was $CD3^+/CD4^-/CD8^-$, and from this subpopulation another subpopulation representing terminally differentiated T cells (TEMRA) that were $CD3^+/CD4^-/CD8^-/CD44^-/CD62L^-$. Representative flow cytometry images from this subpopulation of $CD3^+/CD4^-/CD8^-$ T cells separated by CD62L and CD44 are shown in 2c. The percentages of these various subpopulations present in treated mice were determined. The percentage of CD4–CD8-terminally differentiated T cells (TEMRA) was not significantly increased in PD-1 Ab-treated mice or with PAS monotherapy; however, when PAS100 or PAS250 was combined with PD-1 Ab the CD4-CD8-TEMRA cells increased ($p \leq 0.05$) (Fig. 2d).

The percentage of gamma–delta T cells in the CD4–CD8–TEMRA subpopulation of mice treated with PAS100, PAS250, or the combinations of PAS100 or PAS250 with PD-1 Ab were increased approximately 40% compared to that of PBS-treated controls or PD-1 Ab-treated mice (Supplementary data Fig. 1a). The percentage of NKT cells in the CD4–CD8–TEMRA population was less than 10% in the control PBS-treated mice and the percentage of these cells also significantly changed in mice vaccinated with PAS compared to PBS controls (Supplementary data Fig. 1b).

PAS vaccination and lymphocyte PD-1 expression

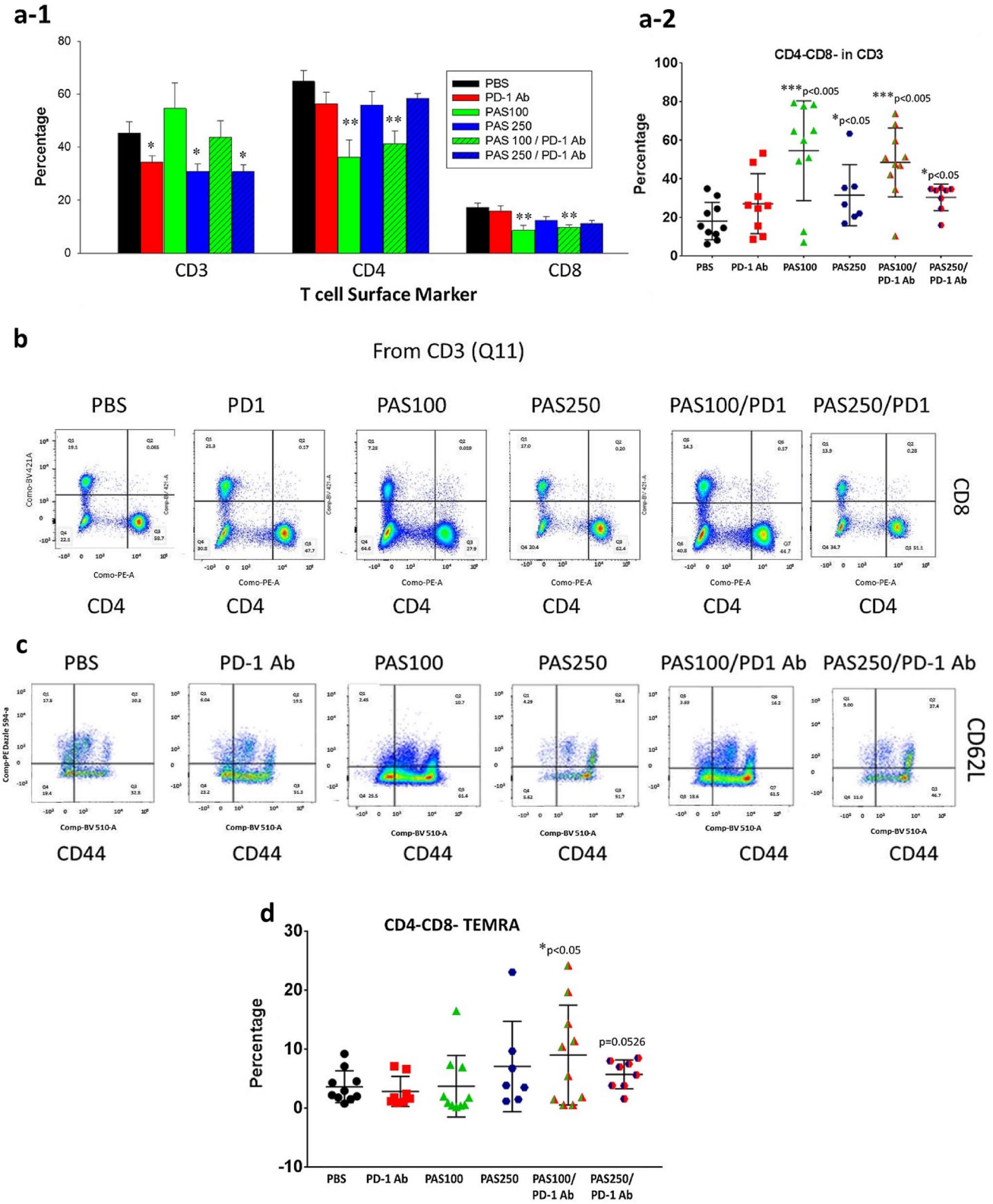
T cells isolated from mouse spleens were analyzed for PD-1 surface ligand expression using flow cytometry. The percentage of PD-1 positive T cells increased by 31.3% and 24% in mice immunized with PAS250 or PD-1 Ab, respectively, compared to PBS controls (Supplementary data Fig. 1c); however, these changes were not significant ($p = 0.053$ for PAS250; $p = 0.192$ for PD-1Ab). The percentage of PD-1 positive T cells was also specifically analyzed in the CD4-CD8-TEMRA cells and was not different from PBS controls.

PAS vaccination induces anti-gastrin antibodies

As expected, PAS elicited a humoral response with generation of titers of anti-gastrin that were at least 90% higher than those in unvaccinated controls (Supplementary data Fig. 2). Mice utilized for this experiment were from the second experiment. There was no difference found in the magnitude of antibody immune response in the mice that were immunized by the i.p. route (PAS100 and PAS100/PD-1 Ab) compared to the mice immunized by the s.c. route (PAS250 and PAS250/PD-1 Ab). These data confirm that vaccination with PAS elicits both a T-cell and a humoral immune response.

PAS100 immunization induces the selective release of cytokines from T-lymphocytes

Isolated splenic T-lymphocytes obtained from mice treated with i.p. injections of PBS, PD-1 Ab or PAS were examined for sensitization to the gastrin peptide by flow cytometry to



determine if they were activated T cells capable of cytokine release. Indeed, T cells from PAS vaccinated mice released cytokines including interferon-γ (INFγ; Fig. 3a-1, a-2), Granzyme-B (granzyme; Fig. 3b-1, b-2), Perforin (Fig. 3c-1, c-2), and tumor necrosis factor-α (TNFα; Fig. 3d-1, d-2). Figure 3 shows that independent groups of T cells isolated

◂**Fig. 2** **a** Effects of PAS, PD-1 Ab, and the combination on splenic mononuclear cells in mice. **a-1** Percentage comparisons of total CD3+, CD4+, and CD8+populations from spleen lymphocytes of mice in each group are shown. **a-2** Percentage of CD3/CD4–CD8 cells from individual mice are shown with the mean and standard deviation. **b** Representative flow cytometry images of viable T cells (CD3+), separated by CD4 and CD8, from spleen lymphocytes of mice treated with i.p. injections of PBS, PD-1 Ab, PAS100, PAS250, PAS100/PD-1 Ab and PAS250/PD-1 Ab. **c** Flow cytometry images of CD4–CD8 populations in **b** above, separated by CD44 and CD62L are shown. **d** Percentage of individual CD4–CD8–TEMRA cells for each mouse is shown for each treatment group. Statistical comparison to control (PBS) gave significance levels of: $*p<0.05$; $**p<0.01$ as indicated in each panel

from mice vaccinated with PAS100 were indeed activated, because they released cytokines compared to T cells from PBS control treated mice. PAS250 vaccinated mice also elicited a cytokine release yet with not as robust of a cytokine release compared to PAS100 in this ex vivo assay. When T cells of the same population from PAS-treated mice were re-stimulated with gastrin for 6 h in culture (panel on the right), most were re-activated and released even higher quantities of cytokines compared to those T cells that were not re-stimulated with gastrin (panel on left). Significant differences between the T-cell cytokine release for each treatment group without gastrin and compared to the same group with gastrin is show in Supplementary Table 3. Each of the four cytokines analyzed increased greater than twofold with combination therapy compared to PAS100 alone. The cytokine activation analysis confirms that vaccination with PAS stimulates T cells and that these cells specifically react to gastrin.

Animal survival and weights

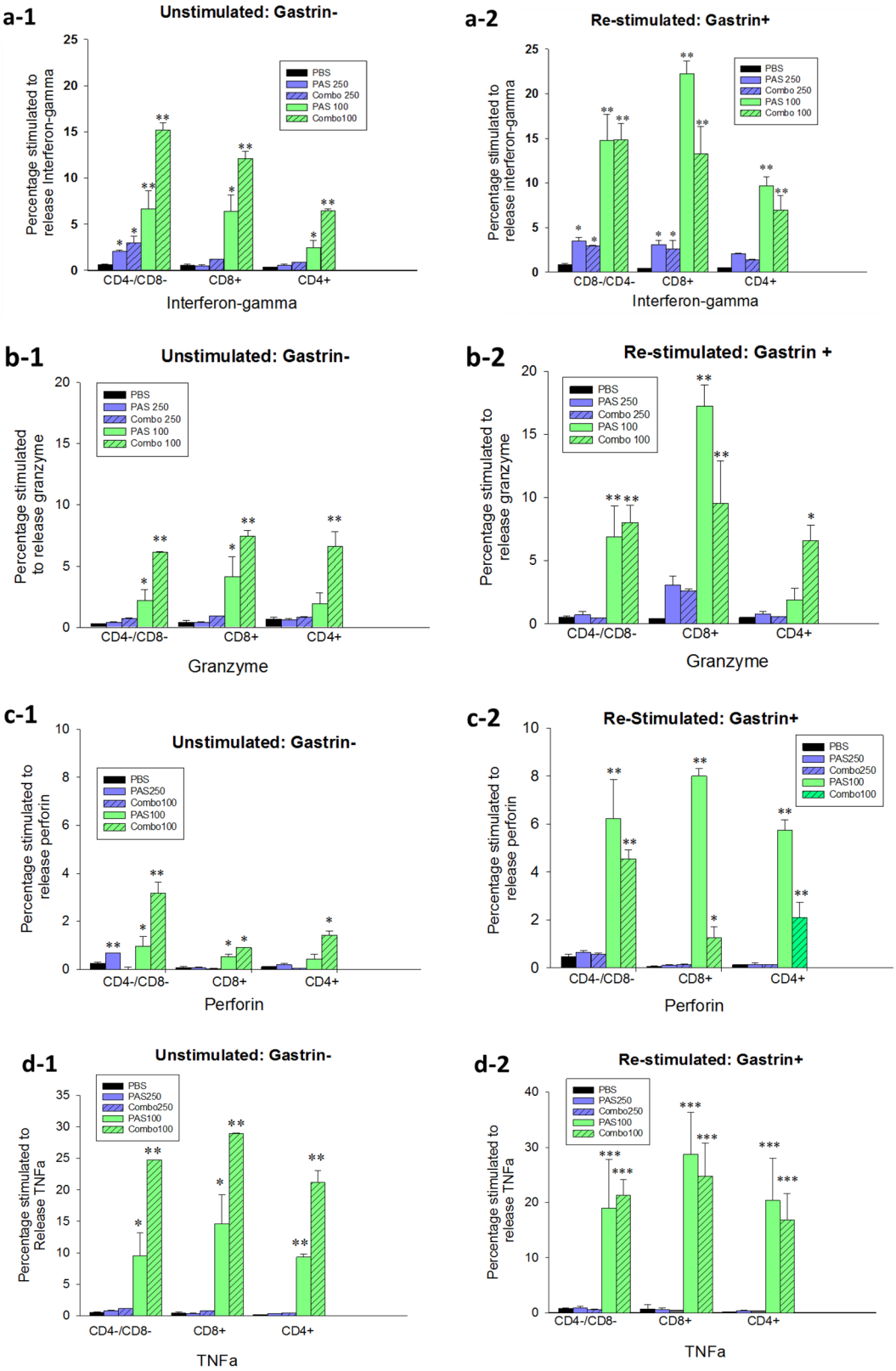
All control and experimental animals were euthanized 31 days after tumor inoculation, because tumors in the PD-1 Ab-treated mice had reached the maximum size allowed by IACUC. In the first experiment, one animal in the PD-1 Ab group died before the start of the experiment by accidently being caught in the cage lid. Three mice in the PAS250-treated groups died prematurely and unexpectedly: PAS250 alone ($N=1$) and PAS250/PD-1 Ab ($N=2$). All three of these mice only had one injection of PAS250 and the two in the combined group had also received two injections of PD-1Ab. These mice did not have large tumors, but it was discovered at autopsy that they had peritonitis. Therefore, these three mice were not used for the splenic immune cells studies for surface receptors or for cytokine re-stimulation. The final tumor measurements (Fig. 4) included $N=10$ mice in the PBS group, PAS100 group and PAS 100/PD-1 Ab group; $N=9$ mice in PD-1 Ab group and PAS250 group, and $N=8$ mice in PAS250/PD-1 Ab group. There were no statistical differences in the animals' body weights among all experimental and control animals (see Supplementary Table 4). In the second experiment one PBS-treated control mouse died in the final experimental week and its tumor size was included in the analysis, and the splenic cells were not used for flow cytometry and there was no evidence of peritonitis.

PAS collaborates with PD-1 Ab to inhibit growth of pancreatic cancer

Tumor volumes were equal in all treatment groups at baseline, which was 1 week after tumor cell inoculation (Fig. 4a). PD-1 Ab monotherapy had no anti-tumor effect and tumor volume in this group was greater than that of control PBS-treated mice (Fig. 4a). Over the treatment period, mice treated with either PAS100 or PAS250 monotherapy or with PAS in combination with PD-1 Ab had smaller tumor volumes compared to those of control PBS-treated and PD-1 Ab therapy alone. The last tumor volume measurements were taken on day 31 (at euthanasia), and at this terminal time point the tumor volumes of the PD-1 Ab-treated mice were significantly larger than all the other treatment groups. Tumor volumes of PAS100-treated mice were not statistically smaller than PBS-treated mice, but the tumor volumes were significantly smaller than those of the PD-1 alone treated mice ($p<0.005$). Tumor volumes in PAS250 monotherapy and PAS250/PD-1 Ab treatments were statistically smaller than the tumors of the PBS-treated mice ($p=0.007$) and the PD-1 Ab-treated mice ($p<0.005$).

Mice treated with PAS250 monotherapy had significantly decreased tumor mass (Fig. 4b) compared to tumor mass of the PBS controls ($p=0.0008$). While monotherapy with either the lower dose of PAS (100 µg) or with PD-1 Ab had no statistically significant effect on tumor mass compared to PBS controls, when PAS (100 µg) was combined with PD-1 Ab there was a synergistic effect that decreased tumor weight (Fig. 4b). Combining PD-1 Ab with PAS250 did not lead to a further reduction in tumor weight compared to that seen with PAS250 alone, but was significantly less than the PBS control tumors ($p<0.005$).

In the second experiment, the tumor volumes were also equal 1 week after tumor inoculation and at the onset of the treatments (Supplementary data, Fig. 3a). Similar to the first experiment, tumors from the mice treated with PD-1 Ab and PAS100 did not change in tumor volume compared to PBS-treated controls; however, PAS 250 monotherapy and PAS100 or PAS250 when combined with PD-1 Ab resulted in smaller tumors. Final tumor weights were also comparable in the second experiment (Supplementary data, Fig. 3b) and the first experiment



(Fig. 4b) with significantly smaller tumors in the mice treated with PAS 250 monotherapy and with PAS100 or PAS250 when combined with PD-1 Ab. These findings confirm the reproducibility of the treatments and results.

◂**Fig. 3** Cytokine stimulation assay. Each panel shows release of cytokines from splenic mouse T cells when treated in vitro with PBS (left) or after re-stimulation with gastrin (right) on the same scale (*y*-axis). Significant changes compared to PBS-treated mice in each group are designated with $*p<0.05$; $**p<0.005$, or $***p<0.0001$. **a-1** Interferon-γ (INFG) cytokine release from various populations of T cells re-stimulated with PBS. **a-2** Results of interferon-γ (INFG) cytokine release from various populations of T cells re-stimulated with gastrin compared to PBS cells also treated with gastrin. **b-1** Granzyme cytokine release from various populations of T cells re-stimulated with PBS. **b-2** Results of granzyme cytokine release from various populations of T cells re-stimulated with gastrin compared to PBS cells also treated with gastrin. **c-1** Perforin cytokine release from various populations of T cells re-stimulated with PBS. **c-2** Results of perforin cytokine release from various populations of T cells re-stimulated with gastrin compared to PBS cells also treated with gastrin. **d-1** Tumor necrosis factor-alpha (TNFa) cytokine release from various populations of T cells treated with PBS. **d-2** Results of TNFa cytokine release from various populations of T cells re-stimulated with gastrin compared to PBS cells also treated with gastrin

PAS collaborates with PD-1 Ab to decrease fibrosis in the tumor microenvironment

Masson's trichrome staining showed extensive fibrosis characteristic of the PDAC tumor microenvironment in tumors from PBS, PAS100, and PD-1 Ab-treated mice (Fig. 5a). Less fibrosis was observed by Masson's trichrome staining in tumors from mice treated with the combination PD-1 Ab and PAS100 therapy (Fig. 5a). Computer analysis confirms statistically significantly decreased collagen staining and fibrosis in tumors from mice in the combination (PAS100/PD-1 Ab) therapy group (Fig. 5b) compared to controls. Similarly, histologic fibrosis in the tumor microenvironment was also observed in the tumors of mice treated with PAS250 (Fig. 5c) but was reduced in tumors of mice treated with PAS250 and PD-1 Ab combination therapy. Computer analysis confirmed that when PAS250 was combined with PD-1 Ab, there was statistically significantly less intratumoral fibrosis (Fig. 5d).

PAS therapy in combination with PD-1 Ab alters tumor infiltrating lymphocytes and tumor-associated macrophages

Immunohistochemical staining shows very few CD8 + TILs in PBS control treated mouse tumors (Fig. 6a, PBS). CD8 + TILs are increased in tumors of all treated mice and were highest in mice receiving combination therapy (Fig. 6a, PAS100 and PD-1 Ab). Computer analysis of CD8 + cells by Image-J is shown in Fig. 6b. CD8 + cells were significantly increased in each treatment group relative to the PBS control with the combined treatment of PAS100/PD-1 Ab increasing CD8 + tumor infiltrating lymphocytes over 100-fold compared to PBS control treated mice.

Numbers of Foxp3 + cells consistent with the tumor immunosuppressive Tregs are high in control tumors from PBS-treated mice (Fig. 6c, PBS). Computer analysis of FoxP3 + cells revealed that treatment with either PD-1 Ab or PAS100 alone had no effect on the quantity of FoxP3 + cells in the tumors. However, tumors of mice treated with the combined therapy of PAS100 and PD-1 Ab had statistically significantly ($p=0.038$) fewer FoxP3 + cells compared to PBS-treated control animals (Fig. 6d). Tumors of mice treated with PAS250 monotherapy and PAS250 in combination with PD-1 Ab also had statistically fewer FoxP3 + staining cells (Fig. 6d).

Representative images from tumors from each treatment group reacted with F4/80 Ab reveal immunoreactivity for tumor-associated macrophages (TAMs) (Fig. 6e). Infiltrating TAMs are abundant within tumors of PBS and PD-1-Ab-treated mice. Statistical comparisons between the treatment groups are shown in Fig. 6f. TAMs are decreased by 19.5% in PAS100 treated mice but this difference is not statistically significant. In contrast, when PAS100 is combined with PD-1 Ab, the number of immunoreactive TAMs decreases by 50% and this decrease is significantly less than that of PBS ($p=0.025$) or compared to PAS100 monotherapy ($p=0.044$). PAS250 monotherapy decreased TAMs by 44% compared to PBS-treated mice and the addition of PD-1 Ab to PAS250 therapy further decreased the number of TAMs to 68.3% compared to PBS controls and this treatment was also significantly greater than PAS250 monotherapy ($p=0.004$).

Discussion

In the current investigation, we found that a peptide vaccine to gastrin, PAS, elicited both a T-cell immune response and a humoral response with neutralizing antibodies to gastrin rendering unresponsive pancreatic cancer tumors susceptible to therapy. PAS vaccination combined with PD-1 Ab therapy induced a synergistic effect to reduce tumor growth and to improve T-cell response when administered concomitantly at doses that had resulted in no effect when administered as monotherapy. Most cancer vaccines and monoclonal antibodies for cancer therapeutics target tumor-associated antigens or neoantigens on the cell surface of cancers [35]. PAS is a unique vaccine, because it selectively targets gastrin, a growth-promoting ligand that activates the CCK-B receptor and is over expressed in certain GI cancers, including pancreatic cancer.

PAS, in addition to eliciting a humoral response with neutralizing gastrin antibodies, also resulted in a significant cellular immune response with the activation of T-lymphocytes. This cellular immune response was noted with PAS monotherapy and was independent of immune checkpoint Ab treatment. Our study showed that the activated T cells

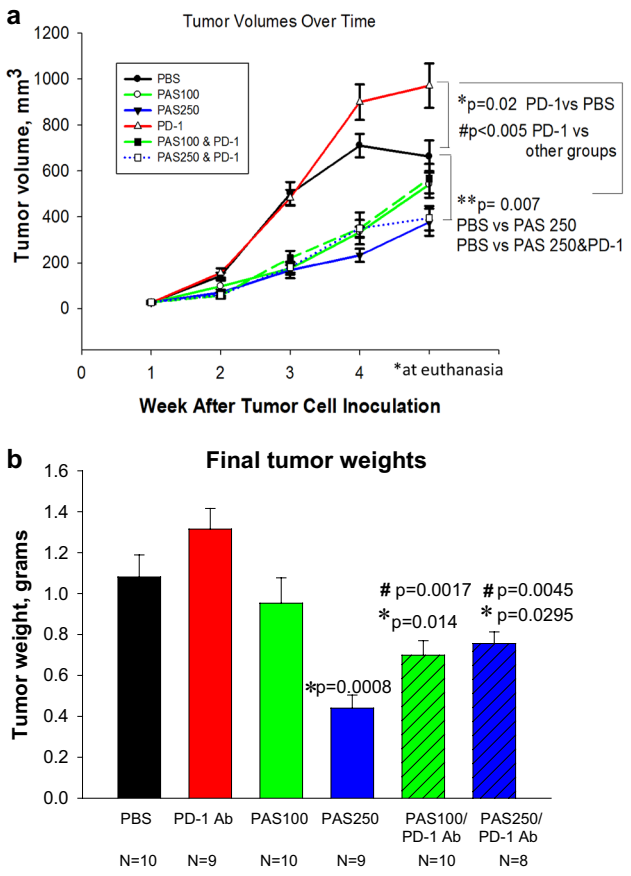


Fig. 4 Tumor volumes over time and final tumor weights. **a** Mean tumor volumes and standard error of the mean are plotted for each weekly assessment over time and a final measurement was taken on day 31 at euthanasia prior to removal of tumors by dissection. Tumor volumes were equal at baseline in all treatment groups. Significant values for the last measurement are shown on the figure. **b** Mean tumor weights at necropsy in all the mice are shown, and the number of animals per group is shown below the x-axis Significance levels are indicated in the figure with $*p$ = significant compared to PBS, $\#p$ = significant compared to PD-1 Ab

responded to gastrin by releasing activated cytokines. This finding supports the conclusion that PAS vaccine was successful in inducing a target-specific T-cell response to the ligand gastrin.

Furthermore, we found that PAS100 and PAS250 combination therapy with PD-1 Ab-activated CD4– and CD8–TEMRA cells that are important in T-cell-mediated tumor death and memory. Recent studies in other malignancies have shown that increased tissue-resident memory T cells represent a new subset of long-lived memory T cells that are associated with prolonged survival in cancer subjects [36]. Another population of CD4–CD8 cells has recently been described that express T-cell receptor γδ (gamma–delta cells) [37]. These γδ-T cells can have direct anti-tumor effects that are mediated by lysing the tumor through the perforin–granzyme pathway, providing an early source of inflammatory cytokines such as IFN-γ and TNF-α [38]. Selective flow cytometry detected a high percentage of γδ-T cells in this CD4–CD8 (double negative) population in all mice treated with both doses of PAS monotherapy or PAS in combination with PD-1 Ab. NKT cells are another population of CD4–CD8 lymphocytes, and the loss of NKT cells has been shown to promote growth of pancreatic cancer [39]. The percentage of NKT cells in the CD4-CD8-TEMRA population were indeed higher in mice vaccinated with PAS. This feature of PAS vaccination resulting in increased γδ and NKT cells was unique to PAS vaccination and the addition of PD-1 Ab therapy did not further increase these subpopulations of T cells. Overall, these results demonstrated the novel finding that PAS vaccination activates both humoral immunity with neutralizing anti-gastrin antibodies and memory T cells.

Our study showed that the activated T cells responded to gastrin by releasing cytokines. This finding supports the conclusion that PAS vaccine was successful in inducing a target-specific T-cell response to the ligand gastrin. All four of the cytokines tested increased in the CD4–CD8 population of cells in the PAS-treated mice supporting the activation of these T cells in response to gastrin in the vaccination. The higher dose of PAS250 was less effective at cytokine re-stimulation in the ex vivo environment. Although this phenomenon has been associated with T-cell exhaustion [40], it is unlikely that this is the reason in our investigation, since in vivo the PAS250 dose was more efficient in inhibiting tumor growth. The blunted cytokine release may also represent evidence of over stimulated immune response.

One remarkable finding in our study was that PAS alone or in combination with PD-1 Ab therapy resulted in significant modification of the tumor microenvironment with decreased fibrosis and changes in the tumor immune cell signatures. The fibrosis remodeling of the tumor microenvironment that was observed in tumors of mice treated with the combined regimen may have been due to the decrease in TAMs but may also have been due to the anti-gastrin antibodies elicited by PAS vaccination. Fibroblasts [26] and pancreatic stellate cells [25] have been shown to express CCK receptors, and when these receptors are activated by exogenous gastrin or autocrine-produced gastrin by the tumor, they become activated and deposit intratumoral collagen. By reducing circulating gastrin with PAS vaccination, there may be less activation of the CCK receptors on tumor fibroblasts resulting in decreased fibrosis. The decreased fibrosis may have then allowed for the migration and influx of CD8 + lymphocytes and the lack of the inhibitory FoxP3 + Tregs. Interestingly, a significant influx of CD8 + tumor infiltrating lymphocytes was seen with both PAS and PD-1 Ab monotherapies, suggesting that each of these agents are capable, separately, to elicit this partial

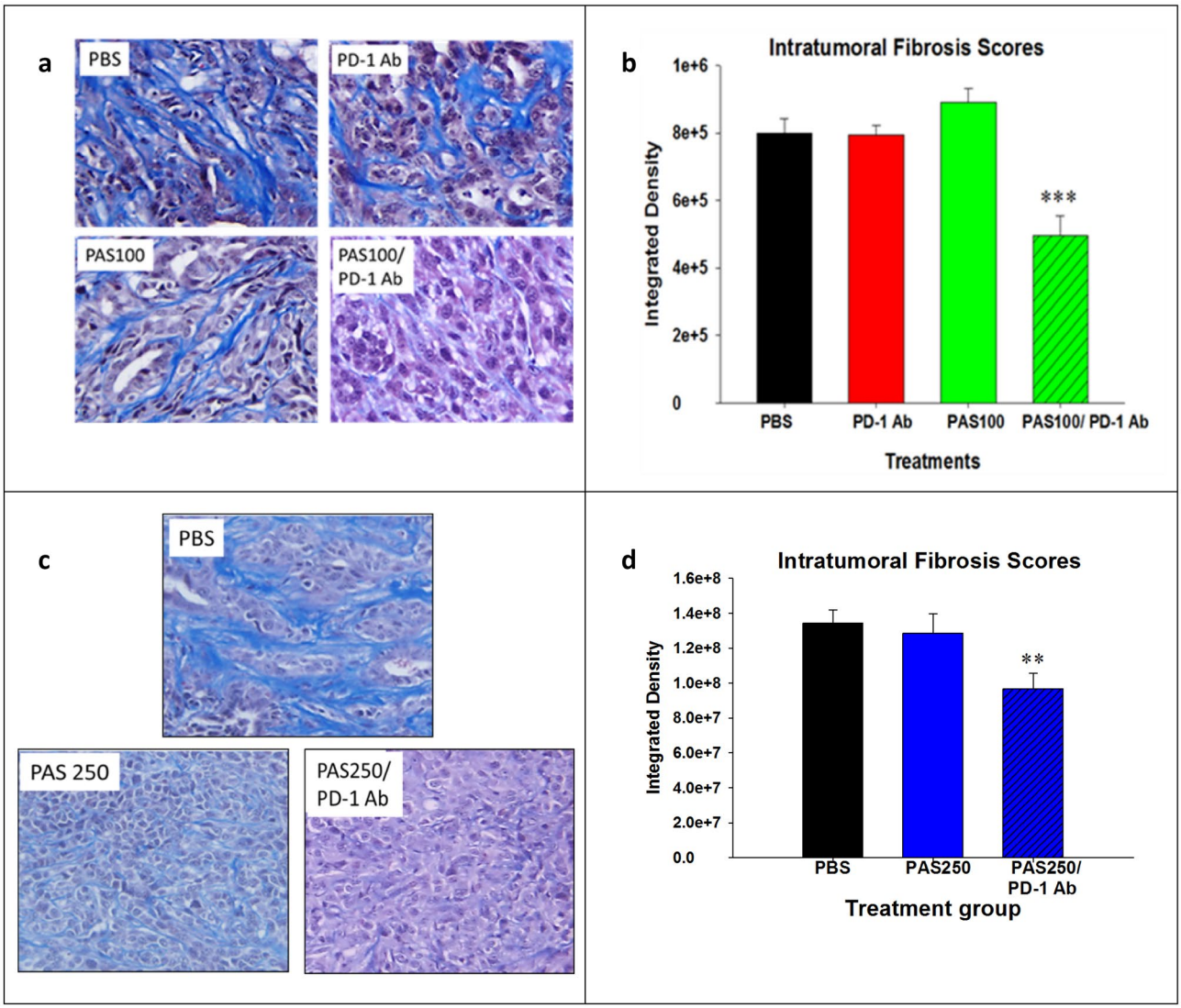


Fig. 5 Intratumoral fibrosis. **a** Representative histologic sections of tumors (Objective 20X) reacted with Masson's trichrome staining from PBS, PD-1 Ab, PAS100 and PAS100 in combination with PAS100 are shown. **b** Computer analysis quantification demonstrated significantly lower fibrosis in mice treated with the combination of PAS100 and PD-1 Ab. ***$p < 0.005$ compared to PBS and $p < 0.001$ compared to PAS100. **c** Representative images (Objective 20X) of Masson's trichrome staining of tumors from mice treated with PBS, PAS250, or PAS in combination with PD-1 Ab are shown. **d** Computer analysis quantification demonstrated significantly lower fibrosis in mice treated with the combination of PAS250 and PD-1 Ab. **$p < 0.01$

immune response but that it was not sufficient to effect tumor size or fibrosis.

A greater number of CD8 + lymphocytes within tumors has also been associated with prolonged disease-free survival and overall survival in pancreatic cancer [41]. Vaccines that elicit a T-cell response may have the capability to potentiate the efficacy of immune checkpoint Ab therapy [42]. Alternatively, Tregs are thought to be immunosuppressive and to impede immune response. Studies have shown that a low dose of cyclophosphamide given prior to therapy may decrease the population of tumor infiltrating Tregs [43]. In the ELIPSE trial using GVAX to treat subjects with advanced pancreatic cancer, patients were pretreated with cyclophosphamide in an effort to decrease the tumor infiltrating Tregs and to enhance therapy [44]. Cyclophosphamide has also been shown to augment responses to PD-1 antibodies when administered prior to therapy [45]. In our study, the mice were not primed with cyclophosphamide, but the combination therapy with PAS and PD-1 Ab significantly decreased the number of immunosuppressive Tregs. These studies support the finding that PAS vaccination activates T cells and alters the immune cell signature within tumors resulting in a more immune sensitive tumor.

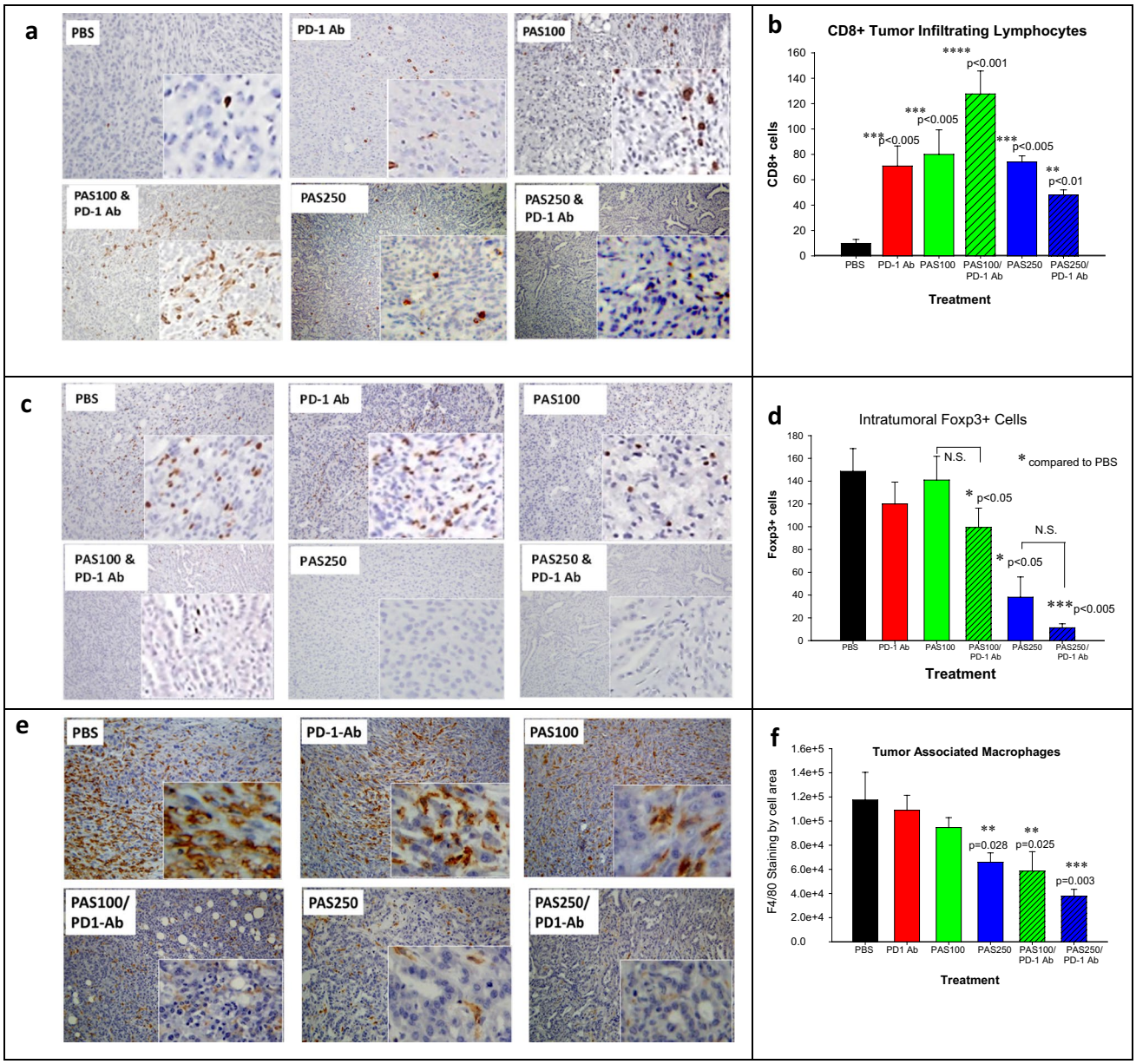


Fig. 6 Tumor infiltrating lymphocytes and tumor-associated macrophages. **a** Immunohistochemistry for CD8 + lymphocytes is shown from tumors representing each treatment group (objective 10X) and with a magnified insert for each treatment. **b** Analysis of cell counts demonstrates that CD8 + intratumoral lymphocytes are rare in tumors of control mice treated with PBS. CD8 + tumor infiltrating lymphocytes are significantly increased in mice treated with PD-1 Ab, PAS-100, or PAS250 ($p < 0.005$) monotherapy. CD8 + cells are the greatest in mice treated with the combination of PAS-100 and PD-1 Ab (compared to PBS; $p < 0.001$). **c** FoxP3 + immunoreactive tumor infiltrating lymphocytes for each treatment group are shown (Objective 10X) including a magnified insert for each treatment. **d** Analysis and cell counts of FoxP3 + tumor infiltrating lymphocytes demonstrates a significant decrease in cell number in tumors of all PAS-treated mice compared to PBS and PD-1 tumors. **e** A representative image of F4/80 + immunoreactive TAMs from tumors of each treatment group are shown (Objective 10X) with a magnified insert from each figure. **f** Mean values calculated from a computerized analysis of each histologic slide area for TAMs and the standard error of the means are shown

The dosages of PAS (100 and 250 µg) and the dosing regimen that was utilized in our study are comparable to those used in prior preclinical studies and to the doses used in clinical trials with this vaccine. Of interest, the lower PAS dose (100 µg) used in this study did not significantly decrease pancreatic cancer mass when used alone. However, when this dose of PAS was combined with PD-1 Ab, the effect on tumor weight was better than with either single agent. The higher PAS dose (250 µg), even when administered as monotherapy was more

effective in decreasing pancreatic tumor size supporting the growth inhibitory effects of this vaccination in PDAC.

Pancreatic ductal adenocarcinoma remains one of the most recalcitrant tumors with the poorest survival of all solid tumor cancers. Mechanisms to improve therapy and to prolong survival are desperately needed for this malignancy. Our novel investigation herein demonstrated that a highly selective vaccine, PAS, directed toward gastrin, a proliferative driver of pancreatic cancer growth and its characteristic microenvironment may prove to be beneficial in future clinical trials.

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Author contributions JPS, NO, RS, LS and AC conceived and designed research; JPS, JB, HC, and XL performed experiments; JPS, NO, RS, JB, HC, XL, AHK, LS and AC analyzed data; JPS, NO, RS, JB, HC, AHK, LS, and AC interpreted results of experiments; JPS, XL, and HC prepared figures; JPS drafted manuscript; NO, RS, JB, HC, XL, AHK, LS, AC, and JPS edited and revised manuscript; ALL authors approved the content of the final version of manuscript.

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Compliance with ethical standards

Conflict of interest Cato Research, Durham NC has intellectual property rights for PAS. PAS compound was transferred to Georgetown University by a Material Transfer agreement with Vaccicure Limited, Liverpool, UK for this research project. All authors work for Cato Research or Georgetown University.

Ethical approval and ethical standards All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the Georgetown University. The IACUC Protocol Number was 2016-1193 and the protocol approval date was 10/05/2017.

Animal source C57BL/6 mice were purchased from Charles River Laboratories (Maryland).

Cell line authentication Murine pancreatic cancer cells, mT3, were a gift from the Tuveson lab (Cold Spring Harbor, NY). Authentication of mT3 murine cells was performed by the investigator that developed the cells [33] and further IMPACT-III testing was performed by IDEXX BioResearch (Columbia, MO) to ensure the cells were pathogen-free.

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