Intralesional injection of Rose Bengal augments the efficacy of gemcitabine chemotherapy against pancreatic tumors

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Abstract

Background

Chemotherapy regimens that include the utilization of gemcitabine are the standard of care in pancreatic cancer patients. However, most patients with advanced pancreatic cancer die within the first 2 years after diagnosis, even if treated with standard of care chemotherapy. This study aims to explore combination therapies that boost the efficacy of standard of care regimens in pancreatic cancer patients.

Methods

In this study, we used PV-10, a 10% solution of rose bengal, to induce the death of human pancreatic tumor cells in vitro. Murine in vivo studies were carried out to examine the effectiveness of the direct injection of PV-10 into syngeneic pancreatic tumor cells in causing lesion-specific ablation. Intralional PV-10 treatment was combined with systemic gemcitabine treatment in tumor-bearing mice to investigate the control of growth among treated tumors and distal untreated tumors. The involvement of the immune-mediated clearance of tumors was examined in immunogenic tumor models that express ovalbumin (OVA).

Results

In this study, we demonstrate that the injection of PV-10 into mouse pancreatic tumors caused lesion-specific ablation. We show that the combination of intralional PV-10 with the systemic administration of gemcitabine caused lesion-specific ablation and delayed the growth of untreated distal tumors. We observed that this treatment strategy was markedly more successful in immunogenic tumors that express the neoantigen, OVA, suggesting that the combination therapy enhanced the immune clearance of tumors. Moreover, the regression of tumors in mice that received PV-10 in combination with gemcitabine was associated with the depletion of splenic CD11b+Gr-1+ cells and increases in damage associated molecular patterns HMGB1, S100A8, and IL-1α.

Conclusions

These results demonstrate that intralional therapy with PV-10 can enhance the efficacy gemcitabine against pancreatic tumors.

Introduction

The intralional injection of PV-10 can induce the destruction of locally treated tumors and simultaneously induce a systemic immune response that promotes the regression of distal, untreated tumors[1, 2]. PV-10 is a solution of the xanthene dye, rose bengal disodium that is currently being
investigated in multiple clinical trials as an anti-cancer agent for multiple malignancies including cutaneous melanoma (NCT02557321) and metastatic liver cancer (NCT00986661)[3, 4].

Previous reports from our group have demonstrated that the direct injection of PV-10 into murine melanoma tumors can completely eliminate treated lesions, but also promote the regression of uninjected bystander lesions in the skin and lungs[1]. We demonstrated that the release of the damage associated molecular pattern (DAMP), high mobility group box 1 (HMGB1), from PV-10 treated tumors induced the activation of dendritic cells (DCs) which subsequently primed anti-tumor T cell responses in lymph nodes. Moreover, treatment with PV-10 in melanoma patients resulted in increased levels of HMGB1 in the serum, which was associated with improved anti-tumor activity of circulating T cells. Thus, in addition to its direct ablative properties, PV-10 is effective at inducing DAMP release from tumors which can augment anti-tumor immune responses.

The clearance of tumors after treatment with radiotherapy and chemotherapy agents is dependent on the induction of immunogenic cell death and the release of DAMPs[5]. However, the immunologic consequences of DAMP release can have disparate effects on anti-tumor immunity. Indeed, HMGB1 promotes the cross-presentation activity of DCs required for T cell priming[6]. Yet, HMGB1 also promotes the accumulation and immunosuppressive capacity of myeloid derived suppressor cells (MDSCs)[7, 8]. Similarly, the DAMP heat shock protein 70 (Hsp70) can prevent the translocation of peptide-major histocompatibility complexes (pMHC) and promote the production of interleukin 10 (IL-10), which subsequently suppresses anti-tumor T cell responses[9, 10]. In contrast, Hsp70 also promotes natural killer (NK) cell-mediated cytotoxicity of tumor cells [11]. Thus, the characterization of the DAMP release and its effects on the immune system are necessary to understand therapeutic responses to anti-cancer agents.

Despite the marked improvement in therapeutic options for development of multiple tumor types over the past decade, pancreatic cancer has remained difficult to treat and only 4% of patients live beyond 5 years after their initial diagnosis[12–14]. Furthermore, frontline treatments that include the utilization of gemcitabine have short-term benefits, but ultimately lead to chemoresistance and disease progression[15–18]. The robust therapeutic resistance of pancreatic cancer is due in part to the architecture of the tumor and the frequency of disseminated disease at the time of clinical presentation. For instance, the pancreatic tumor stroma can promote T cell exclusion and also harbors a highly immunosuppressive microenvironment, which induces T cell dysfunction[19–22]. Moreover, tumor-infiltrating T cells are localize to stroma elements of tumors and are spatially distant from pancreatic tumor cells[23]. Thus, it is imperative to develop new therapeutic strategies to augment standard of care practices with a goal of promoting anti-tumor immunity against local and distant pancreatic tumors.

In this study, we investigated the efficacy of intralesional (i.l.) PV-10 treatment in combination with gemcitabine in pancreatic tumor models. We demonstrate that PV-10 can effectively augment the efficacy of gemcitabine against murine pancreatic tumors.
Results

PV-10 kills pancreatic tumor cells in vitro

We first determined whether PV-10 could effectively kill pancreatic tumor cells. We cultured murine Panc02 tumor cells with varying concentrations of PV-10 for 24hrs and determined that a concentration of 200 µM was the most effective at inducing cell death as determined by Annexin-V and DAPI positivity (Fig. 1A). We next cultured a panel of human pancreatic tumor cell lines (CFPAC1, MiaPaca2, Panc-1, and SU8686) and murine Panc02 in media without PV-10, 50 µM PV-10, or 200 µM PV-10. We found that 200 µM PV-10 was highly effective at inducing cell death as indicated by Annexin-V and DAPI double-positive cells, albeit the frequency of dead cells varied amongst individual tumor cell lines. Similarly, the frequency of live cells (Annexin-V−DAPI) were decreased in all tested tumor cell lines after treatment with 200 µM PV-10. Moreover, PV-10 modestly increased the frequency of Annexin-V+DAPI− and Annexin-V−DAPI+ cells in some tumor cell lines. Additionally, the 50 µM PV-10 increased cell death compared to untreated tumor cells, but at a lower rate in comparison to cells treated with 200 µM PV-10 (Fig. 1B-E).

We next determined that the saturation of PV-10 in tumor cells was associated with increased cell death. Rose bengal excites at 525 nm and emits a fluorescent signal at 570 nm which allowed us to assess tumor cell uptake of PV-10. Notably, the staining intensity corresponded to the frequency of dead cells. We observed that the frequency of dead Panc-1 cells increased after treatment with 200 µM PV-10 and that the staining intensity was higher in comparison to Panc-1 cells treated with 50 µM PV-10 (Fig. 2A). In cells treated with 50 µM PV-10, we identified two distinct populations of tumor cells that had differing intensities of PV-10 staining and uptake (PV-10high and PV-10low). In most tumor cells lines, the frequency of live cells was significantly reduced in PV-10high tumor cells in comparison to PV-10low cells. Moreover, dead cells were almost exclusively contained within the PV-10high population (Fig. 2B-D). Together, these data demonstrate that PV-10 can effectively kill human and murine pancreatic tumor cells.

In vivo efficacy of intralesional PV-10 against murine pancreatic tumors

First, we evaluated whether the release of DAMPs could be associated with tumor regressions in response to PV-10 treatment. TLR4 is a receptor for lipopolysaccharide (LPS), but also for the DAMP, HMGB1. We used a reporter cell line to assess the activation of TLR4. Serum was collected from PV-10 treated mice or control PBS treated mice 24hrs after intralesional (i.l.) injection and cultured with HEK-Blue mTLR4 cells overnight. TLR4 reporter activity was increased in cells exposed to serum from PV-10 treated mice (Fig. 3A). Likewise, the abundance of HMGB1 was increased in the serum of mice 24 hours after PV-10 treatment, suggesting that HMGB1 could activate TLR4 in PV-10 treated mice (Fig. 3B). Next, we assessed the activity of PV-10 in a bilateral model in mice with Panc02 tumors. One tumor was treated with i.l. PBS or PV-10, while the tumor on the opposite flank was left untreated. PV-10 slowed tumor growth in treated tumors but had no effect on bystander uninjected tumors (Fig. 3C-D).
Figure 3. Effect of PV-10 against murine Panc02 tumors in vivo. (A) Serum collected from mice treated with i.l. PBS or i.l. PV-10 were collected 24hrs after treatment. Serum was cultured with HEKBlue-mTLR4 reporter cells overnight. (B) HMGB1 in the sera of mice treated with i.l. PBS or i.l. PV-10. (C) Tumor growth of treated tumors with i.l. PBS or i.l. PV-10. (D) Tumor growth of uninjected tumors implanted on the opposite flank of mice.

**Gemcitabine treatment is enhanced by intralesional PV-10**

PV-10 effectively delayed tumor growth after i.l. injection into Panc02 tumors but did not promote a systemic immune response that could elicit anti-tumor activity in uninjected tumors. We hypothesized that the PV-10 would effectively induce a systemic immune response to pancreatic tumor cells that expressed a highly immunogenic antigen. In mice with a single Panc02 tumor expressing the ovalbumin (OVA) protein, we found that i.l. treatment with PV-10 was as effective as treatment with systemic gemcitabine or the combination of i.l. PV-10 and gemcitabine (Fig. 4A). However, in a bilateral tumor model gemcitabine treatment alone failed to reduce tumor growth in either lesion, suggesting that the increased tumor burden dampened the efficacy of the chemotherapy. In contrast, i.l. PV-10 treatment alone and the combination of i.l. PV-10 with systemic gemcitabine induced the complete regression in 50% and 62.5% of treated tumors respectively (Fig. 4B). In uninjected bystander tumors, tumor growth was effectively delayed in mice that received i.l. PV-10 in one lesion in combination with systemic gemcitabine (Fig. 4C). Together, these data suggest that tumor burden and the immunogenicity of pancreatic tumors affect the efficacy of combinatorial i.l. PV-10 and systemic gemcitabine.

In a single-flank model in mice with Panc02 tumors, we observed that i.l. PV-10 alone delayed tumor growth in a subset of mice in comparison to mice that received i.l. PBS. Moreover, systemic gemcitabine delayed tumor growth, but the combination of i.l. PV-10 with systemic gemcitabine was the most effective at delaying tumor growth (Fig. 5A-B). Next, we harvested tumors at the termination of the experiment and confirmed that mice treated with i.l. PBS and systemic gemcitabine had smaller tumors in comparison to mice that received i.l. PBS alone or PV-10 alone. Moreover, tumors were significantly smaller in mice that received the combination treatment of PV-10 with systemic gemcitabine (Fig. 5C). Thus, these data demonstrate that i.l. PV-10 enhances the efficacy of systemic gemcitabine treatment.

Figure 5. Combination therapy with PV-10 and gemcitabine induces tumor regression. (A) Individual tumor growth curves in mice that received i.l. PBS (top left), i.l. PV-10 (top right), i.l. PBS + i.p. Gem (bottom left), i.l. PV-10 + i.p. Gem (bottom right). (B) Summary of tumor growth curves from (A) (n = 6–8 per group). Data are representative of 2 independent experiments (C) The mass of tumors at the termination of the experiment. Data are a compilation of 2 independent experiments. (n = 16–17 per group).

**Systemic effects of PV-10 and gemcitabine**

Next, we wanted to identify correlates that could explain the enhanced anti-tumor effect of gemcitabine when combined with PV-10. We analyzed the frequency of immune cells within the spleens of mice that received i.l. PV-10 and/or gemcitabine. We identified that the frequency of CD4 or CD8 T cells were
unchanged by either PV-10 or gemcitabine (Fig. 6A). In contrast, gemcitabine effectively reduced the frequency of total CD11b+ myeloid cells in mice that received combination treatment with i.l. PBS or i.l. PV-10 (Fig. 6B). Specifically, gemcitabine reduced the frequency of Gr-1+ cells, while Gr-1− cells comprised a higher proportion of myeloid cells in mice that received gemcitabine (Fig. 6C). Next, we examined the abundance of DAMPs in the serum of mice 9 days after receiving treatment. We identified that S100A8, IL-1α, and HMGB1 were only elevated in mice that received both i.l. PV-10 and gemcitabine (Fig. 7A, C-D); S100A9 and Hsp70 was not significantly altered by PV-10 or gemcitabine (Fig. 7B, E). Furthermore, these DAMPs were only elevated in mice that received both i.l. PV-10 and gemcitabine. Thus, the enhanced anti-tumor activity of combinatorial i.l. PV-10 with systemic gemcitabine is associated with reduced CD11b+Gr-1+ cells and increased S100A8, IL-1α, and HMGB1.

**Discussion**

Combination therapy strategies with gemcitabine have often failed to improve the survival in patients with pancreatic cancer[15–18]. Notably, the addition of nab-paclitaxel has increased the survival of pancreatic cancer patients when treatment is combined with gemcitabine. However, the 2-year survival rate in patients that receive nab-paclitaxel plus gemcitabine is approximately 9% in comparison to 4% who received gemcitabine alone[18]. Thus, there is a potent need to define therapeutic combinations that enhance the durability of clinical responses that ultimately extend the survival of pancreatic cancer patients. With the results described in this study, we believe that it is feasible to combine intralesional therapy with PV-10 with standard of care chemotherapy. We provide evidence that the potentiation of anti-tumor immune responses is necessary for profound tumor growth stabilization and regression. Specifically, treated Panc02 tumors were controlled upon i.l. therapy with PV-10, but untreated bystander tumors were unaffected (Fig. 3C-D). In contrast, in Panc02 tumors that express that immunogenic neoantigen, OVA, we observed that monotherapy with gemcitabine or PV-10 was equally as effective at controlling tumor growth in a single flank model (Fig. 4A). Moreover, PV-10 treatment alone or in combination with gemcitabine was capable of inducing the complete regression of treated tumors, while gemcitabine monotherapy failed to induce complete regressions. This was in contrast to the effectiveness of gemcitabine monotherapy in mice bearing a single Panc02OVA tumor. This suggests that the increased tumor burden in mice with lesions on both flanks diminished the efficacy of gemcitabine, which was overcome when combined with i.l. PV-10. In addition, the rate of tumor growth of untreated bystander tumors was slowed in mice that received PV-10 in combination with gemcitabine versus gemcitabine alone (Fig. 4B-C). This suggests that an immunogenic antigen potentiates the combination of PV-10 and gemcitabine which results in the regression of local and distally untreated tumors.

We next examined the efficacy of PV-10 combination therapy in Panc02 tumors that did not express OVA. We observed that PV-10 treatment alone had a modest effect in reducing tumor growth. Indeed, gemcitabine monotherapy effectively reduced tumor growth. However, the reduction of tumor growth was enhanced in mice that received combination therapy with PV-10 and gemcitabine (Fig. 5). Despite that
Panc02 tumors are less immunogenic than Panc02OVA tumors, we were able to observe a significant improvement in tumor growth control and regression in mice that received combination therapy. Thus, the combination of gemcitabine with PV-10 can induce tumor regression even in less immunogenic tumors.

Treatment with gemcitabine is associated with the depletion of MDSCs and the promotion of tumoricidal activity by tumor-associated macrophages\[24, 25\]. Indeed, we observed that gemcitabine effectively reduced the frequency of bulk CD11b\(^+\) myeloid cells within spleens. However, there was a proportional shift characterized by the reduction of CD11b\(^+\)Gr-1\(^+\) MDSCs and an increase in CD11b\(^+\)Gr-1\(^-\) myeloid cells. This reduction of MDSCs was ultimately associated with reduced tumor growth in mice that received gemcitabine alone or the combination with PV-10. We further investigated systemic changes that could impact the immune system in response to PV-10 treatment. Indeed, PV-10 treatment alone increased the abundance of HMGB1 within 24hrs after injection (Fig. 3B). We and others have shown that HMGB1 is an important mediator of DC activation and promotion of anti-tumor immunity\[1, 6, 26\]. Intriguingly, the increased abundance of HMGB1 and other DAMPs persisted in mice that received PV-10 in combination with gemcitabine (Fig. 7). Specifically, we observed that HMGB1, S100A8, and IL-1\(\alpha\) were increased 9 days after treatment in mice that received PV-10 in combination with gemcitabine. Notably, mice that received the combination therapy exhibited the greatest reduction in tumor growth amongst all experimental groups, suggesting that the increased abundance of DAMPs in circulation is associated with better therapeutic responses. While HMGB1 can promote anti-tumor immune responses, it can simultaneously potentiate tumor cell survival mechanisms\[27, 28\]. Similarly, S100 proteins and IL-1\(\alpha\) appear to have important roles in promoting pancreatic tumor progression. For instance, S100A8 and S100A9 enhances the production of IL-8 in pancreatic tumor cells which could promote the accumulation of immunosuppressive myeloid cells, including MDSCs\[29–31\]. Meanwhile, IL-1\(\alpha\) can enhance the metastatic potential of pancreatic tumor cells by maintaining the constitutive activation of nuclear factor \(\kappa\)-B (NF\(\kappa\)B) and promoting the secretion of hepatocyte growth factor (HGF)\[32, 33\]. Despite the roles of these DAMPs in promoting pancreatic tumor progression, we observed that the increase of these factors was associated with tumor regression in mouse models. Hence, the specific roles of DAMPs that are released by pancreatic tumors after PV-10 treatment remain unclear. Future studies will address the immunological consequences of these DAMPs on promoting anti-tumor immunity in the context of pancreatic cancer.

In conclusion, we demonstrate that intralesional therapy with PV-10 is a feasible strategy to augment therapeutic responses when combined with gemcitabine. Together, the results of this study provide support for future studies to investigate the induction of systemic anti-tumor immune responses after PV-10 treatment.

**Methods**

**Cell lines and cell culture**
Panc02 pancreatic cancer (obtained from ATCC), were cultured in RPMI media supplemented with 10% heat-inactivated FBS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM fresh L-glutamine, 100 mg/ml streptomycin, 100 U/ml penicillin, 50 mg/ml gentamicin, 0.5 mg/ml fungizone (all from Life Technologies, Rockville, MD), and 0.05 mM 2-ME (Sigma-Aldrich, St. Louis, MO). To generate the ovalbumin (OVA) expressing fluorescent Panc02 cell line, cells were exposed to supernatants containing a lentiviral vector comprised of a fluorescent ZsGreen (ZsG) protein and OVA. Upon successful transfection, ZsGreen<sup>hi</sup> tumor cells were subjected to FACS using BD FACSaria. OVA-ZsGreen<sup>hi</sup> tumor cells were passaged <em>in vitro</em> 4 times whereby OVA expression was validated by staining for H2-K<sup>b</sup> bound to SIINFEKL peptide (25-D1.16, BioLegend). CFPAC1, MiaPaca2, Panc-1, and SU8686 cells (obtained from ATCC) were grown and maintained in culture according to supplier guidelines. The cell lines tested negative for mycoplasma contamination. All cell lines were passaged less than 10 times after initial revival from frozen stocks. All cell lines were authenticated using STR profiling in 2018.

**Apoptosis and cell death detection**

Human and murine pancreatic tumor cells were cultured in 12 well plates and grown to ~ 60% confluency. Then, the indicated concentrations of PV-10 were added to media and cells were cultured for 24hrs. Adherent cells were collected by gentle scraping and pooled with non-adherent cells. Cells were washed 3 times in PBS to remove excess PV-10. Washed cells were then stained with Annexin-V APC and DAPI (both from BioLegend) and analyzed on a BD FACSCelesta to determine the frequency of apoptotic and dead cells.

**Mouse models and treatment**

Female C57BL/6 mice (6–8 weeks old) were purchased from Charles River Laboratories. Animal studies were carried out in compliance with ARRIVE guidelines. Mice were randomized before and after tumor implantation when put onto a study that included drug treatment. Panc02 and Panc02OVA-ZsGreen tumor cells (5x10<sup>4</sup>) were implanted subcutaneously into one flank of a mouse to establish a single tumor. To establish a bilateral tumor model, tumor cells were implanted in the opposite flanks. On day 7, a single tumor was treated with intralesional PV-10. Investigators could not be blinded to mice that received PV-10 due to the red staining of the tumor tissue and surrounding skin that was apparent within days after injection. Gemcitabine (60 mg/kg) was injected intraperitoneally twice per week for 2 weeks. Mice were housed at the Animal Research Facility of the H. Lee Moffitt Cancer Center and Research Institute. Tumor growth measurement studies were concluded when > 30% of an experimental group was found dead or required humane euthanization. Mice were humanely euthanized by CO<sub>2</sub> inhalation and cervical dislocation according to the American Veterinary Medical Association Guidelines. Mice were observed daily and were humanely euthanized if a solitary subcutaneous tumor exceeded 300 mm<sup>2</sup> in area, evidence of ulceration, or mice showed signs referable to metastatic cancer. All animal experiments were approved by the Institutional Animal Care and Use Committee and performed in accordance with the U.S. Public Health Service policy and National Research Council guidelines.

**Detection of DAMPs in mouse serum**
Blood was collected at the termination of experiments. Blood specimens were centrifuged at 2000xg for 10 minutes at room temperature to separate serum from other blood content. The abundance of HMGB1 was determined by HMGB1 ELISA (IBL International); HSP70 and IL-1α were determined by Human/Mouse/Rat Total HSP70/HSPA1A DuoSet IC ELISA and Mouse IL-1 alpha/IL-1F1 Quantikine ELISA Kit (both from R&D Systems, a Biotechne brand); S100A8 and S100A9 were determined by Mouse Magnetic Luminex Assay (R&D Systems, a Biotechne brand) and analyzed on the Luminex 100 (LuminexCorp).

**Assessment of TLR4 activity by HEK-Blue mTLR4 reporter cell line**

Cryopreserved HEK-Blue mTLR4 cells (InvivoGen) were thawed, washed with pre-warmed medium (DMEM, 4.5 g/l glucose, 10% (v/v) FBS, 100U/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL Normocin, 2 mM L-Glutamine) and then transferred to a 25 cm² tissue culture flask containing 5 mL media. HEK-Blue mTLR4 cells were grown to 70-80% confluence and passaged twice before use in determining TLR4 activity. 2.5x10⁴ HEK-Blue mTLR4 cells were seeded in wells of a flat-bottom 96 well plate in medium containing 1X HEK-Blue Selection and 16% mouse serum taken from Panc02 tumor bearing mice treated with i.l. PBS or i.l. PV-10. Cultures were incubated for 18hrs and TLR4 activity was determined by the detection of secreted embryonic alkaline phosphatase (SEAP) using a spectrophotometer at 655 nm.

**Flow cytometry**

Tissues were prepared for flow cytometric analysis as previously described [34]. Briefly, spleens were harvested under sterile conditions and were homogenized by forcing the tissue through 100 µm cell strainers using the plunger from a syringe. Single-cell suspensions were prepared, and red blood cells were removed using red blood cell lysis buffer (BioLegend). The resulting suspension was passed through a 70 µm cell strainer and washed once with PBS. Cells were resuspended in to a concentration of 0.5-1x10⁶ cells/mL for flow cytometric analysis in FACS Buffer containing PBS, 5% fetal bovine serum, 1 mM ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich), and 0.1% sodium azide (Sigma Aldrich). Cell viability was measured by staining cell suspensions with ZombieNIR (BioLegend). Prior to surface staining, cells were incubated with Fc Shield (TonboBiosciences) for murine specimens. For surface staining of murine specimens, cells were stained in FACS buffer with the following antibodies: CD3 (145-2C11), CD4 (GK1.5), CD8 (53 – 6.7), CD11b (M1/70), Gr-1 (RB6-8C5) (all from BioLegend). Flurochromes that overlapped with the emission spectra of PV-10 were not used in this study. Cells were acquired by FACS Celesta (BD Biosciences), and the data were analyzed with FlowJo (Tree Star).

**Statistical Analysis**

Graphs were generated using GraphPad Prism software. Graphs represent mean values with SEM. P values were calculated in each respective figure where statistical tests were indicated. For mouse-tumor growth studies, tumor growth curves are shown as mean with SEM and significance was determined by
2-way ANOVA and Sidak's multiple comparison's test. Mice were randomized after tumor cell implantation into respective treatment groups. For all other experiments, data were compared using either an unpaired 2-tailed Student's t-test corrected for multiple comparisons by a Bonferroni adjustment or Welch's correction. *=P < 0.05; **=P < 0.01; ***=P < 0.001; ****=P < 0.0001; ns = not significant.

**Declarations**

**Ethics approval and consent to participate**

Animal studies were carried out in compliance with ARRIVE guidelines. Mice were humanely euthanized by CO₂ inhalation and cervical dislocation according to the American Veterinary Medical Association Guidelines. Mice were observed daily and were humanely euthanized if a solitary subcutaneous tumor exceeded 300 mm² in area, evidence of ulceration, or mice showed signs referable to metastatic cancer. All animal experiments were approved by the Institutional Animal Care and Use Committee and performed in accordance with the U.S. Public Health Service policy and National Research Council guidelines.

**Consent for Publication**

Not Applicable.

**Competing Interests**

Not Applicable.

**Author Contributions**

P.I. Wrote the manuscript, prepared figures 1-7, performed experiments for figures 1, 2, 6, and performed data analysis and interpretation. J.M., A.M., S.A., M.B., J.B. S.K. performed experiments for Figures 3-7. J.E.M, A.A.S, and S.P-T. designed experiments, and provided data analysis and interpretation. S.P-T. provided critical revision of the article and final approval of the version to be published. All authors reviewed the manuscript.

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Data Availability Statement

The datasets generated and/or analyzed during the current study are not publicly available because this study does not contain any large datasets that require the submission to a data repository. Data are available from the corresponding author on reasonable request.

References


