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Supplemental Information

Polymer-Mediated Inhibition of Pro-invasive Nucleic Acid DAMPs and Microvesicles Limits Pancreatic Cancer Metastasis

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Supplementary Materials and Methods:

Patient Blood Collection

With approval from the Duke University Institutional Review Board, blood was collected from patients with pancreatic cancer after presentation with localized (early stage) or advanced disease and—in patients with early stage disease—at various time points in their treatment course, including before and after neoadjuvant (preoperative) chemoradiation therapy, intraoperatively during surgical resection, and one week postoperatively. Neoadjuvant chemoradiation therapy consisted of 45-50 Gy of external beam radiation therapy delivered over approximately 5 weeks with concurrent oral capecitabine as a radiation sensitizer [62]. Patients with advanced disease had undergone a variety of therapies including chemotherapy and/or radiation therapy. Blood was collected into BD SST Vacutainer tubes, and the sample was spun at 1500 rpm for 5 min. The supernatant was collected and aliquoted as patient sera for use.

Reagents

Generation 3.0 polyamidoamine dendrimer solution (PAMAM-G3) was purchased from Sigma Aldrich Cat. # 412422). The TLR agonists CpG ODN 2006, CpG ODN 1826, ODN 2088, Poly I:C, LPS, and R848 were purchased from Invivogen.

Human PC Cell Lines

Human BxPC3, Panc1, MiaPaCa2, and AsPc1 pancreatic cancer cell lines were purchased from ATCC. The following cell growth media were used for each cell line: BxPC3 and AsPC1 (RPMI 1640 plus 1% L-glutamine, 1% penicillin-streptomycin, 10% heat inactivated FBS), Panc1 (DMEM-high glucose plus 10% non-heat inactivated FBS), and MiaPaCa2

(DMEM-high glucose plus 10% non-heat inactivated FBS, 2.5% equine serum, 1% penicillin-streptomycin).

Generation of KPC4580P Murine PC Cell Line

The KPC4580P murine PC cell line- derived from the KPC genetic mouse model of pancreatic ductal adenocarcinoma (a gift of Jen Jen Yeh MD)- was generated in the following manner. In compliance with the University of North Carolina IACUC protocols, mice with Cre-inducible Kras and p53 mutations, Cre-inducible luciferase expression, and a pancreas specific Cre promoter were bred to obtain LSL-$Kras^{G12D/+}$; LSL-$Trp53^{R172H/+}$; $PDX1^{Cre/+}$; LSL-$ROSA26^{Luc/+}$ mice. This combination promotes the development of pancreatic ductal adenocarcinoma, as published before [46], with the addition of luciferase expression [63], which allows for easier follow up of the mutated cells. After PCR confirmation of the alleles, the mice were followed for disease progression and body condition. Tumor size was determined by either palpation or ultrasound. Once the tumor reached the maximum allowed size or the mouse had reached a humane endpoint, the mouse was euthanized and tumors were excised. Upon removal, part of the tumor was cut into approximately 3mm pieces and rinsed with PBS containing 5X penicillin/streptomycin (500 IU/mL and 500µg/mL). The tissue was then incubated for 1 hour in a 0.1 U/ml collagenase and 0.8 U/ml dispase solution. After incubation, dissociation media was removed, remaining tumor pieces were minced further and the tissue was resuspended in DMEM, 10% FBS and 1X penicillin/streptomycin and seeded onto tissue culture treated plates. The cells were incubated at 37˚C in a humidified chamber with 5% CO_2 and standard tissue culture protocols were used for their culture. Trypsin-EDTA was used for subculturing the cells. Cre-mediated recombination was confirmed by detection of the HINDIII restriction site in

the mutant Kras allele [64], and by detection of bioluminescence by the addition of D-luciferin to live cells. Cell growth media for the KPC4580P cell line consisted of DMEM-F12 media plus 10% non-heat inactivated FBS.

Quantification of Cell-Free DNA, Nucleosome, and HMGB1 Levels

Total DNA was isolated from sera using the DNA Blood Mini Kit (Qiagen) and quantified using the PicoGreen Staining Kit (Life Technologies). Nucleosome levels were quantified using the Cell Death ELISA Plus Kit (Roche). HMGB1 levels were quantified using an ELISA kit (Chondrex).

TLR Activation Assays

HEK-Blue TLR 4 and 9 reporter cell lines were purchased from Invivogen and activation in response to control agonists or human sera was determined according to the manufacturer's instructions. These cells stably co-express a TLR gene and an NFkB-inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene that can be monitored using SEAP detection media. Briefly, these cells were plated in 96-well, clear-bottom, flat-bottom plates at a density of 100,000 cells per well with at least 5 wells per condition. The cells were then treated for 18-24 hours with either 1) media alone, 2) a control agonist for each given TLR (LPS [1 μg/mL] for TLR 4 and CpG ODN [5 μM] for TLR 9), 3) cancer patient sera [3.5 μL], 4) normal human sera [3.5 μL], 5) media alone + PAMAM-G3 [20 μg/mL], 6) control agonist + PAMAM-G3 [20 μg/mL], 7) cancer patient sera [3.5 μL] + PAMAM-G3 [20 μg/mL], or 8) normal patient sera [3.5 μL] + PAMAM-G3 in a final volume of 100 μL. After this incubation period, the cell supernatant was collected and mixed with Quantiblue (Invivogen) at a 60:40 vol:vol ratio and

incubated for 5 hours at 37C in a new 96 well plate, after which time absorbance at 650 nm was measured using a Spectramax i3 plate reader (Molecular Devices).

Microparticle and Exosome Isolation from Cultured PC cell lines and PC Patient Sera

KPC-4580P cells were cultured to confluence in T-175 flasks (Corning), at which time media was replaced with complete medium supplemented with exosome-depleted FBS instead of normal FBS. After 72 hours, the supernatant was collected and centrifuged at 4000g for 5 min to deplete cell debris. The residual supernatant was centrifuged at 20,000g for 20 min at 4C to pellet microparticles (MPs). The supernatant was removed and saved for exosome isolation. The MP pellet was washed with PBS (-/-) and re-centrifuged at 20,000g for 20 minutes at 4C. This supernatant was discarded and the final MP pellet was resuspended in 1mL of PBS, aliquoted and stored at -80 C. The supernatant from the initial MP spin was centrifuged at 100,000g for 2 hours at 4C to pellet exosomes. After discarding the supernatant, the exosome pellet was washed with PBS and this mixture was re-centrifuged at 100,000g for 2 hours at 4C. The supernatant was discarded and the exosome pellet was resuspended in 500 μL of PBS, aliquoted and stored at -80C. The protein concentration of the MPs and exosomes was quantified via Pierce BCA assay (Thermo-Fisher). To isolate MPs from human sera, sera was similarly centrifuged at 20,000 g for 20 minutes at 4C, supernatant was discarded, and resuspended in PBS.

Transwell-Matrigel Invasion Chamber Assays

Transwell-Matrigel invasion chambers were purchased from Corning and assays were performed according to the manufacturer's instructions. Briefly, the invasion chambers were first incubated with serum-free media in the top and bottom chambers at 37C for a minimum of 1.5

hours to allow the matrigel to solidify and the two chambers to equilibrate in preparation for the addition of PC cells and various agonists and/or inhibitors such as CpG ODN, PC cell line derived MPs and exosomes, PAMAM-G3, and ODN 2088. The human (BxPC3, MiaPaca2, PANC-1, AsPC-1) or mouse (KPC-4580P) PC cell lines were trypsinized, resuspended, and diluted in serum free media. After aspiration of media from both chambers, cells were plated in the top chamber at a density of 50,000 cells/well (500 μL final volume) in the presence of serum free media alone or various combinations of agonists and/or PAMAM-G3 [20 μg/mL] or ODN 2088 [100 μM]. Agonists tested included CpG ODN [5 μM], PC cell line derived MPs or exosomes (isolated as described above), PC patient sera or normal human sera (50 μL), or mouse sera (45 μL). 750 μL of complete media was added to the bottom chambers. All conditions were tested in duplicate. The plated invasion chambers were incubated at 37C for 24 hours, after which time media was aspirated from the top chambers. The top chambers were then removed and placed into a new 24-well plate preloaded with 1 mL of 10% formaldehyde per well to fix the invaded cells on the bottom surface of the chamber. After fixation for 10 minutes, the top chambers were then removed from the formaldehyde and placed into a new 24-well plate preloaded with 1 mL of PBS (-/-) per well for 1 minute for washing purposes. The top chambers were then placed onto absorbent pads and residual PBS was wiped from the inside of the top chambers using a cotton swab. 10 μL of crystal violet solution (5% w/v crystal violet, 25% v/v methanol) was added to the membranes and allowed to stain for 5 minutes. Crystal violet was from Sigma Aldrich. The chambers were then washed thoroughly in deionized water and residual crystal violet was wiped from the inside of the chambers using a cotton swab. The membranes were then imaged using an inverted light microscope (Olympus IX50) at 4X magnification. 10 random images of each membrane were taken using an eyepiece camera (Dino-Eye AM7023B).

The cells in the images were then counted using an ImageJ algorithm unique for each cell line (http://imagej.net/Particle_Analysis).

Analysis of Nuclear NFkB Translocation

KPC-4580P cells were plated at $2x10^6$ cells per plate in 35 mm plates overnight and BxPC3 cells were plated at $2x10^5$ cells per plate in 35 mm plates overnight. The cells were then treated with serum-free media alone, CpG ODN (1826 or 2006) [5 μM], or CpG [5 μM] and PAMAM-G3 [20 μg/mL] for 1 hour. Cell nuclei were then isolated using a nuclear isolation kit (Active Motif) using the manufacturer's guidelines. The protein content of each nuclear extract was quantified and standardized using a Protein Quantification Kit (Active Motif). The nuclear extract was then used to quantify nuclear translocation of NFkB via ELISA. For the murine nuclear extracts, nuclear translocation of p50 NFkB was quantified using the Pierce p50 Transcription Factor Assay Kit (Thermo Fisher) using the manufacturer's instructions. For the human nuclear extracts, nuclear translocation of p65 NFkB was quantified using the p65 TransAM ELISA (Active Motif).

Analysis of Cell Viability

In order to replicate conditions used for the invasion chamber assays, Panc1 or KPC4580P cells were seeded overnight in 96-well assay plates at a density of 50,000 cells/well. Panc1 cell were then treated with CpG ODN 2006 [5 μM], PAMAM-G3 [20 μg/mL], or media (4 wells per treatment group). After 24 hours, cell viability was measured according to the Cell Titer Glo Luminescent Cell Viability Assay (Promega), according to the manufacturer's instructions. KPC4580P cells were treated with CpG ODN 1826 [5 μM], KPC4580P-derived MPs (100

ng/μL), KPC4580P-derived exosomes (50 ng/μL), PAMAM-G3 [20 μg/mL], 1% Triton X-100, or media (12 wells per treatment group). After 24 hours, cell viability was measured according to the Cell Titer Glo Luminescent Cell Viability Assay (Promega), according to the manufacturer's instructions.

ELISA for Detection of PAMAM-G3 Binding to MPs

The binding of MPs and exosomes to PAMAM-G3 was quantified using a custom-designed ELISA assay. MPs and exosomes were isolated from KPC4580P PC cell cultures as described above. 96-well high binding, flat bottom plates (Corning) were coated overnight at 4C with PAMAM-G3 diluted into PBS (-/-) at varying concentrations (0, 0.2, 2, or 20 μg/mL) in duplicate rows (100 μL/well). The wells were washed 3 times with PBS and incubated for 2 hours at RT with blocking buffer (PBS plus 5% bovine serum albumin and 0.05% Tween-20). After washing 3 times with PBS, the wells were incubated with varying concentrations of MPs or exosomes for 2 hours at RT. After washing 3 times with PBS, the wells were then incubated with either the polyclonal goat IgG against murine tissue factor (AF3178, R&D Systems) for MP detection or the monoclonal mouse IgG antibody against phosphatidylserine (05-719, Millipore) for exosome detection. Both antibodies were used at a 1:1000 dilution in PBS plus 1% BSA and 0.05% Tween-20) and incubated for 1 hour at RT. After another 3 washes with PBS, the wells were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 hour at RT. The wells were washed again 3 times with PBS. At this point TMB substrate (100 μL) was added to each well, and stop solution (100 μL) was added after 15 min. Absorbance at 450 nm was measured by a Spectramax i3 plate reader (Molecular Diagnostics). The binding of PC patient-derived MPs to PAMAM was quantified analogously. MPs were isolated from pooled

patient sera by centrifugation at 20,000g for 20 min at 4C, and the ELISA procedure was followed as described above using the anti-phosphatidylserine primary antibody for MP detection.

Syngeneic Murine Model of Pancreatic Cancer Metastasis

All animal experiments were performed in compliance with Duke IACUC protocols. Briefly, for metastasis studies, KPC4580P luciferase expressing, murine PC cells (described above) were surgically implanted into the spleens of 12 week old female C57BL6 mice. Starting on Day 2 after tumor cell implantation, mice were treated twice per week with either intraperitoneal PAMAM-G3 (20 mg/kg) or saline for 4 weeks or until humane endpoints were reached, at which time the mice were sacrificed. Liver and spleen were harvested from each mouse for assessment of gross organ weight and *ex vivo* quantification of bioluminescent intensity (IVIS Kinetic). Just prior to sacrifice, caudal vena cava blood was collected from each mouse for analysis of blood cell counts (Heska HemaTrue blood analyzer), plasma cfDNA (described above), nucleosome levels (described above), HMGB1 levels (described above), or chemistry (performed by Duke DLAR Veterinary Diagnostic Lab), or for serum invasion assays (described above). For PAMAM-G3 toxicity studies, 12 week old female C57BL6 mice were treated twice per week with either intraperitoneal PAMAM-G3 (20 mg/kg) or saline for 3 weeks, at which time the mice were sacrificed. Just prior to sacrifice, submandibular blood was collected from each mouse for blood cell count analysis or plasma chemistry analysis as described above.

A more detailed description of the above procedure for tumor cell implantation now follows. KPC4580P cells were cultured to confluence, trypsinized, washed three times with sterile PBS, and resuspended in 1:1 (vol/vol) media:matrigel for a total injection volume of 50

μL (100,000 cells/mouse) and kept on ice prior to surgery. Matrigel matrix (growth factor reduced, phenol red free) was purchased from Corning. Anesthesia was induced with 3 to 5% isoflurane inhaled in a sealed chamber 3-5 minutes or until effect. After induction, each mouse was moved to dedicated prep station and placed in dorsal recumbency on a heating pad, while maintaining anesthesia with 1-3% isoflurane delivered by a nose cone. Lubricant was applied to the eyes to prevent dessication. Hair was removed from the ventral abdomen by shaving. Buprenorphine (0.10 mg/kg) was administered subcutaneously for analgesia. The surgical site on the abdomen was prepped with a povidone/iodine skin scrub followed by an alcohol rinse with sterile gauze, a total of three times. At this time, the mouse was moved to a dedicated surgical station, with anesthesia maintained with 1-3% isoflurane delivered by a nose cone and body temperature maintained at approximately 37°C with a Physitemp TCAT-2DF. The mouse was then placed in right lateral recumbency. Using sterile microscissors, an approximately 1.5 cm longitudinal incision was made in the skin, 1-cm left, lateral of midline, slightly medial to the spleen. Next a 1.5-cm incision was made in the abdominal musculature, mirroring the overlying superficial incision. The spleen was located using forceps and gently removed from the abdominal cavity. The cell suspension was carefully injected into the spleen using a sterile 29G needle syringe. Once the cells were injected, the needle was kept in the spleen for 60 seconds so that the matrigel solidified, ensuring that the cells did not leak into the abdominal cavity. The needle was removed, and the spleen was gently internalized using blunt tipped forceps. The abdominal musculature was closed using a 6-0 absorbable suture with an interrupted stitch. Prior to skin closure, the mouse was dosed with 2 mg/kg bupivacaine via syringe between the abdominal and cutaneous layers to ensure proper analgesia. The overlying skin was closed using a skin staple. At this point the mouse was removed from the inhaled anesthetic. The mouse was

allowed to recover in its cage with free access to food and water. All mice received subcutaneous buprenorphine (0.1 mg/kg) 24 hours after surgery. Additionally, if any mice displayed signs of pain such as hunching, they were provided with subcutaneous buprenorphine every 12 hours as needed. In 7-10 days, once the surgical wound healed, the skin staples were removed.

Statistical Analysis

All data were plotted and analyzed using Graphpad Prism or JMP software. Data were all tested for normality prior to statistically analyses. If normally distributed, differences between groups were compared using a two-tailed student's t-test. If the data were not normally distributed, as in the case of the *in vivo* organ bioluminescence and gross weight data, statistical analysis between groups was performed using the Kruskal Wallis test.

Supplementary References

63. Safran, M, Kim, WY, Kung, AL, Horner, JW, DePinho, RA, and Kaelin, WG (2003). Mouse reporter strain for noninvasive bioluminescent imaging of cells that have undergone Cre-mediated recombination. *Molecular imaging* **2**: 297-302.
64. Aguirre, AJ, Bardeesy, N, Sinha, M, Lopez, L, Tuveson, DA, Horner, J*, et al.* (2003). Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma. *Genes & Development* **17**: 3112-3126.

Supplementary Figure 1

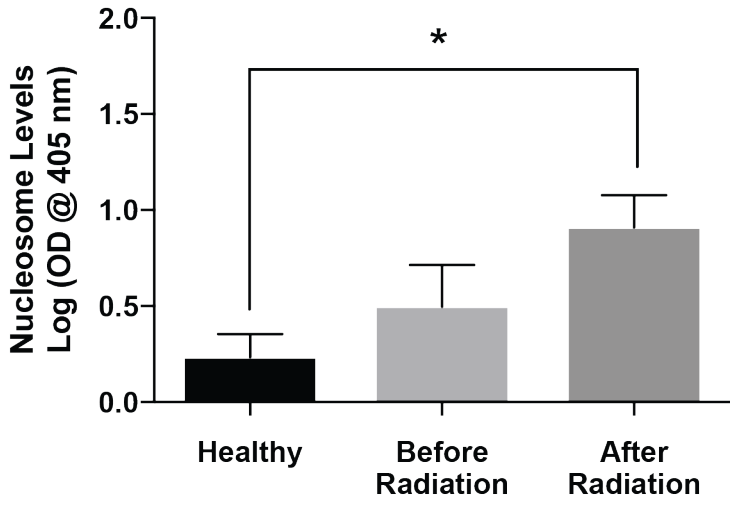

Figure S1. Circulating nucleosome levels are elevated in pancreatic cancer patients and further elevated after chemoradiation therapy (CRT). Serum nucleosome levels in healthy individuals and pancreatic cancer (PC) patients with localized, early stage disease before and after CRT (N = 8 for all groups), as measured by ELISA. * denotes $p < 0.05$ by two-tailed t-test. Raw data were log-transformed in order to satisfy normality requirement for parametric analysis by two-tailed t-test.

Supplementary Figure 2

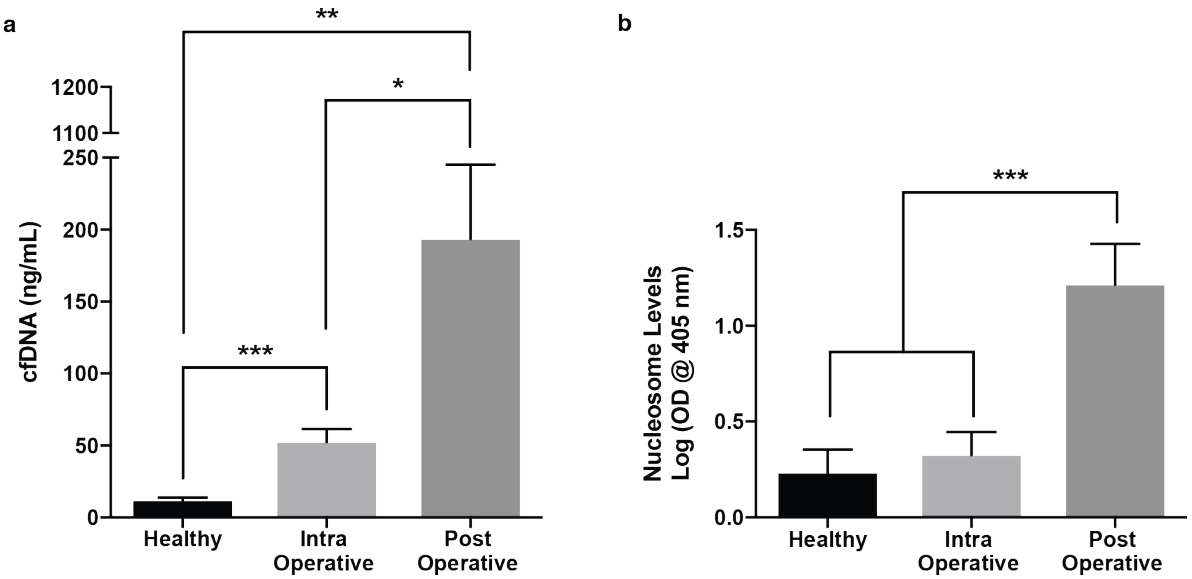

Figure S2. Elevation in serum cfDNA and nucleosome levels in pancreatic cancer patients during and after surgery. Serum cfDNA (a) and nucleosome (b) levels in healthy individuals and pancreatic cancer (PC) patients before and after surgical resection therapy, as measured by Picogreen staining and nucleosome ELISA respectively. N = 6 for all samples. ***, **, and * denote $p < 0.001$, $p < 0.01$, and $p < 0.05$ respectively by two-tailed t-test.

Supplementary Figure 3

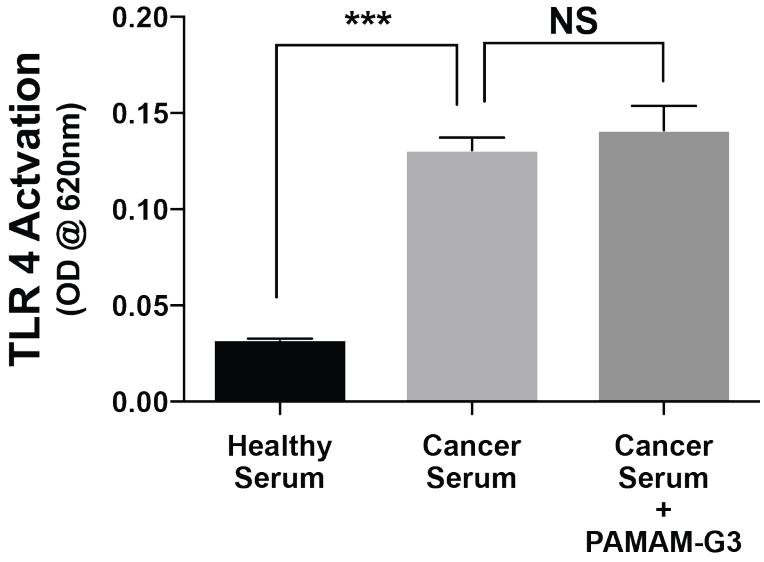

Figure S3. Pancreatic cancer patient serum activates TLR 4 but these effects are not inhibited by PAMAM-G3. TLR 4 activation by healthy human or pancreatic cancer (PC) patient sera in TLR 4 specific reporter cells, and effect with PAMAM-G3 (20 μg/mL) treatment. Individual experiments were performed with serum from two different PC patients and figure depicts a single representative experiment. *** and NS denote $p < 0.001$ and "not significant" respectively by two-tailed t-test.

Supplementary Figure 4

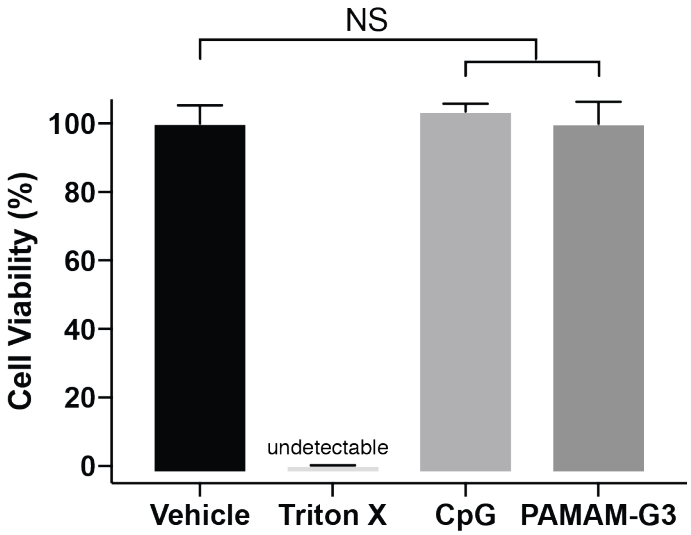

Figure S4. Treatment of pancreatic cancer (PC) cells with CpG ODN or PAMAM-G3 does not affect cell proliferation. Cell viability as measured by Cell-Titer Glow assay was determined after incubation of KPC4580P murine PC cells with media (vehicle), 1% Triton X-100, CpG ODN 1826 (5 μM), or PAMAM-G3 (20 μg/mL), for 24 hours. NS = not significant by two-tailed t-test.

Supplementary Figure 5

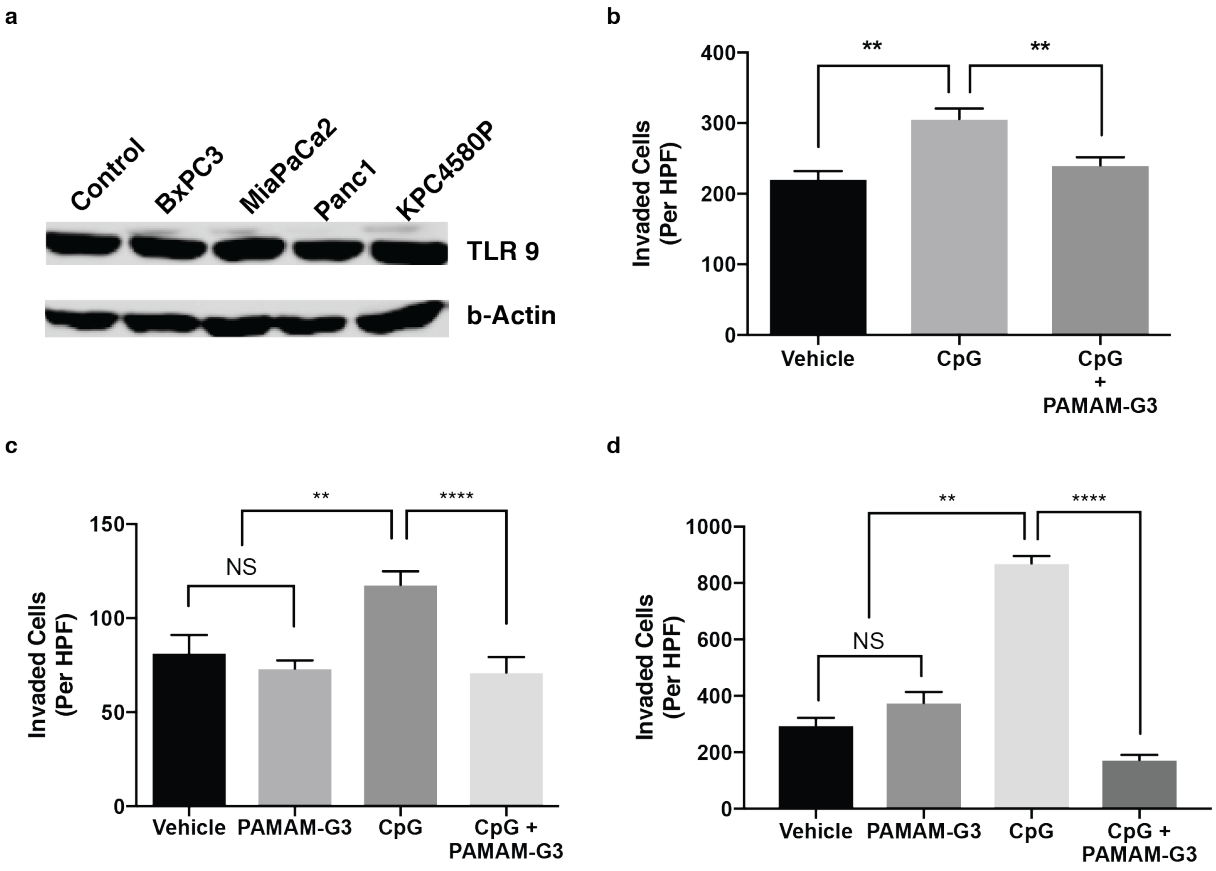

Figure S5. PAMAM-G3 inhibits CpG ODN induced invasion in human and murine pancreatic cancer cell lines. (a) Western blot expression of TLR 9 by a panel of human (BxPC3, MiaPaCa2) and murine (KPC4580P) pancreatic cancer cell lines. **(b-c)** Invasion of BxPC3 (b) and MiaPaCa2 (c) cell lines upon treatment with human TLR9 specific agonist CpG ODN 2006 (5 μM) in the absence or presence of PAMAM-G3 (20 μg/mL). **(d)** Invasion of KPC4580P cells upon treatment with the murine TLR9 specific agonist CpG ODN 1826 (5 μM) in the absence or presence of PAMAM-G3 (20 μg/mL). Effect of PAMAM-G3 (20 μg/mL) alone on cell invasion is also shown. ****, **, and NS denote $p < 0.0001$, $p < 0.01$, and "not significant" by two-tailed t-test. All invasion experiments were repeated at least three times and each bar graph depicts mean ± SEM of a single representative experiment.

Supplementary Figure 6

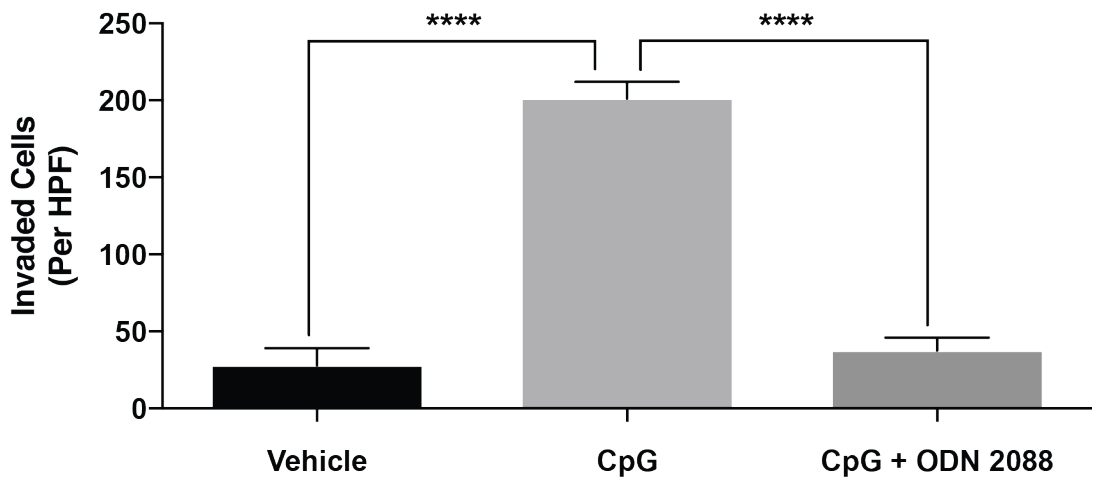

Figure S6. TLR 9 inhibitor, ODN 2088, inhibits CpG ODN induced invasion in pancreatic cancer cells. Invasion of KPC4580P cells upon treatment with human TLR 9 specific agonist CpG ODN 2006 (5 μM) in the absence or presence of the human TLR 9 specific oligonucleotide inhibitor ODN 2088 (100 μM). **** denotes $p < 0.0001$ by two-tailed t-test. Invasion experiment was repeated at least three times and each bar graph depicts mean ± SEM of a single representative experiment.

Supplementary Figure 7

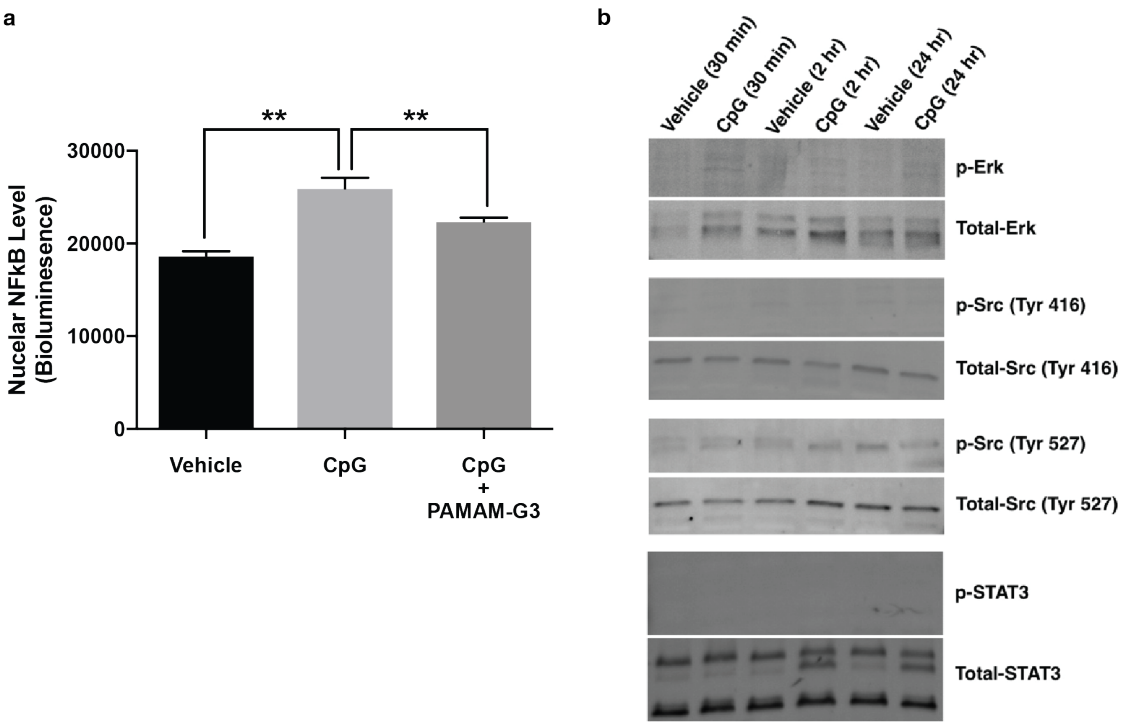

Figure S7. Addition of CpG ODN to PC cells increases nuclear translocation of p50 NFkB which is restored to baseline levels with PAMAM-G3 treatment, but does not activate the ERK, Src, and STAT3 signaling pathways. (a) Effect of CpG ODN 1826 (5 μM) treatment, alone or in combination with PAMAM-G3 (20 μg/mL), on nuclear translocation of NFkB in KPC4580P pancreatic cancer cells, as quantified by ELISA luminescent readout. Experiment was repeated three times and bar graph depicts mean ± SEM of a single representative experiment. ** denotes $p < 0.01$ by two-tailed t-test. (b) Effect of CpG ODN 1826 (5 μM) or vehicle (media) treatment on activation of the ERK, Src, and STAT3 signaling pathways as measured by phosphorylated ERK, Src (Tyr-416 or Tyr-527), and STAT3 detection by Western Blot in cell lysates collected at 30 min, 2 hr, or 24 hr post-treatment. Experiment was repeated three times and figure depicts a single representative experiment.

Supplementary Figure 8

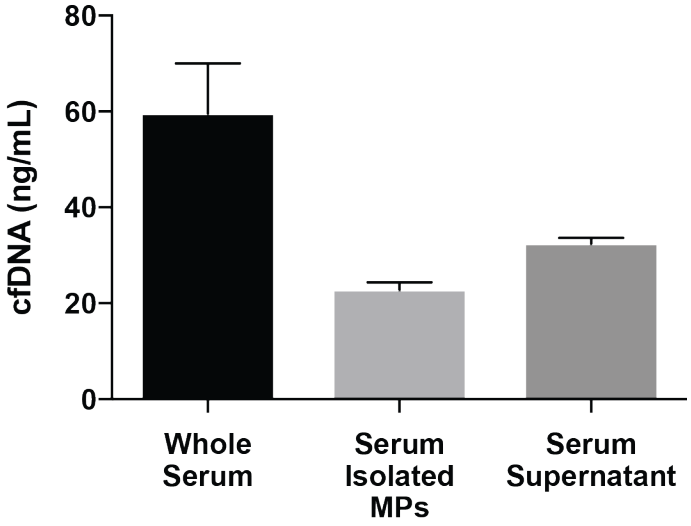

Figure S8. cfDNA in pancreatic cancer patient sera exists in both microvesicle bound and unbound forms. Pancreatic cancer patient serum was fractionated into microparticles and supernatant, and cell free DNA was quantified via PicoGreen staining after isolation with Qiagen's DNA Blood Mini Kit.

Supplementary Figure 9

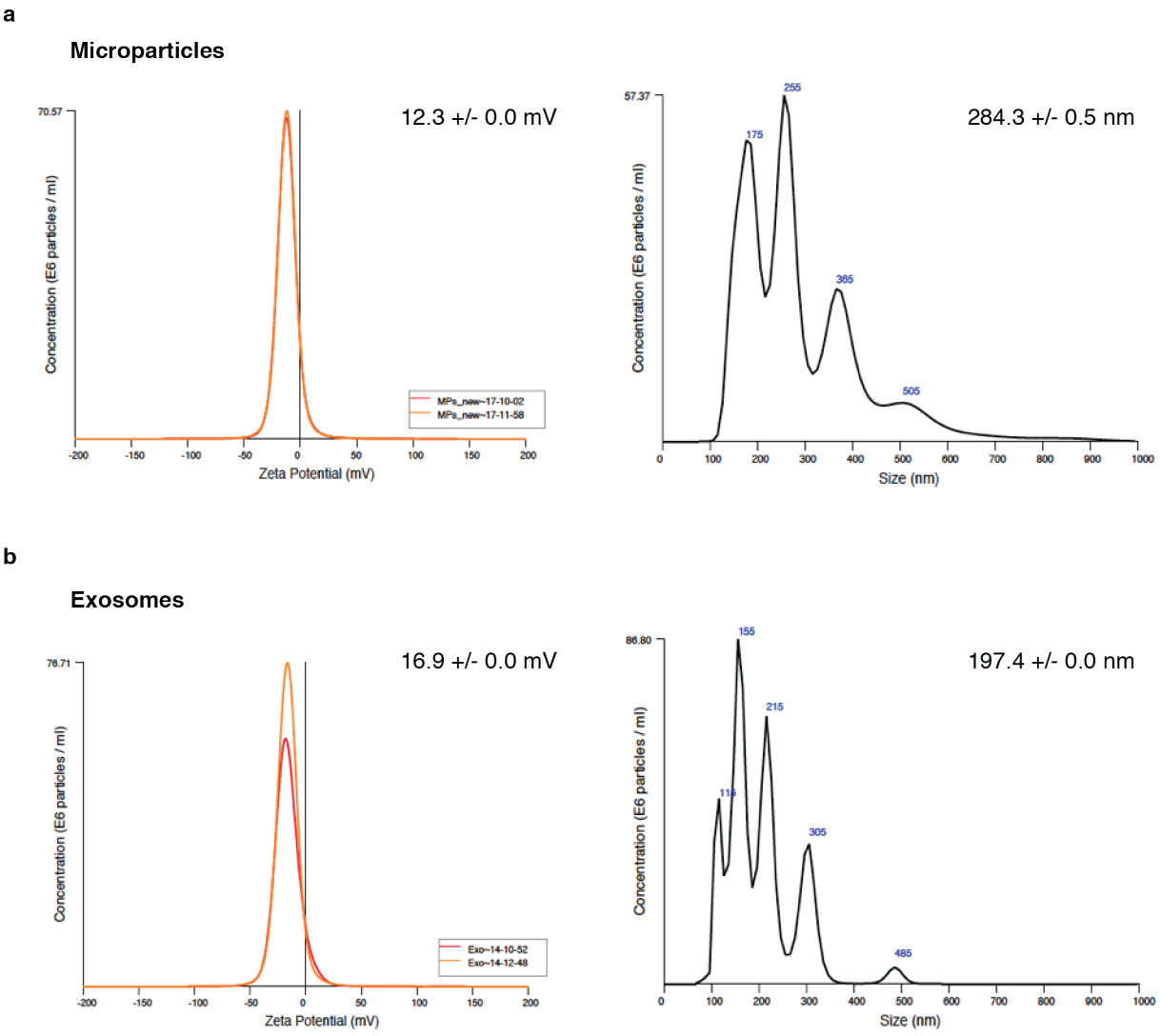

Figure S9. Pancreatic cancer derived microparticles and exosomes bear an electronegative surface charge. KPC4580P cell line derived microparticles (MPs) (a) and exosomes (b) were counted, sized, and analyzed for their surface charge using a Nanosight Zeta Potential Platform.

Supplementary Figure 10

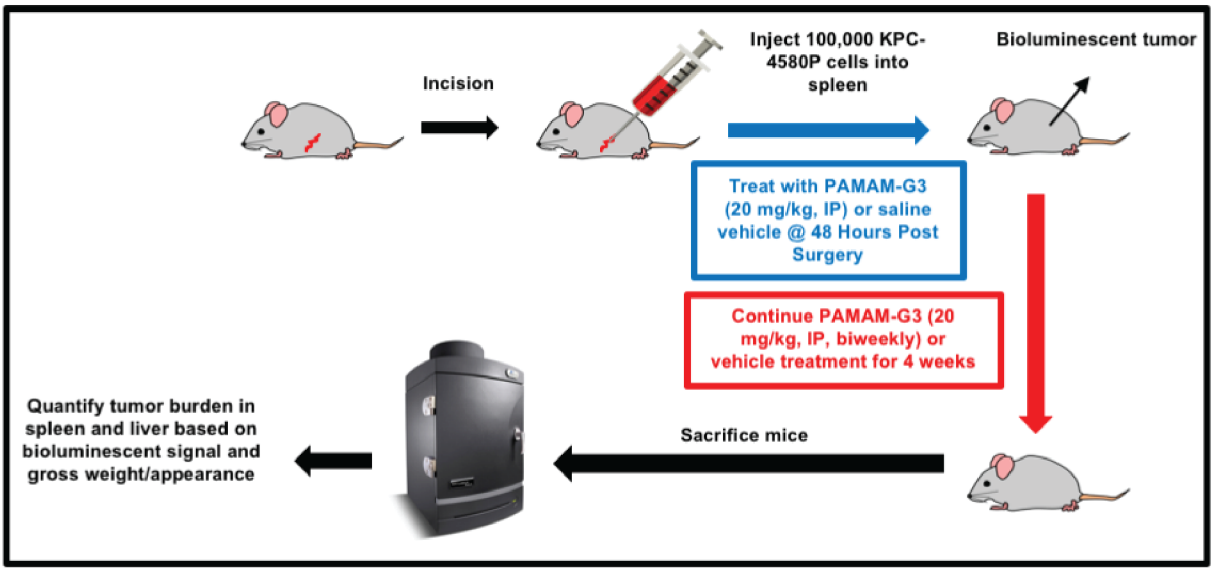

Figure S10. Experimental schematic of *in vivo* studies to evaluate the effect of PAMAM-G3 (G3) in a syngeneic murine model of pancreatic cancer metastasis. C57BL6 mice surgically implanted with 10^5 KPC4580P cells in their spleens reliably develop liver metastasis within 3-4 weeks. Groups of 25 mice were treated twice weekly with intraperitoneal injections of PAMAM-G3 (20 mg/kg) or saline vehicle starting 48 hours after tumor cell implantation. Primary (spleen) and metastatic (liver) tumor burden were quantified by measuring tumor specific *ex vivo* organ bioluminescence and weight after 4 weeks.

Supplementary Figure 11

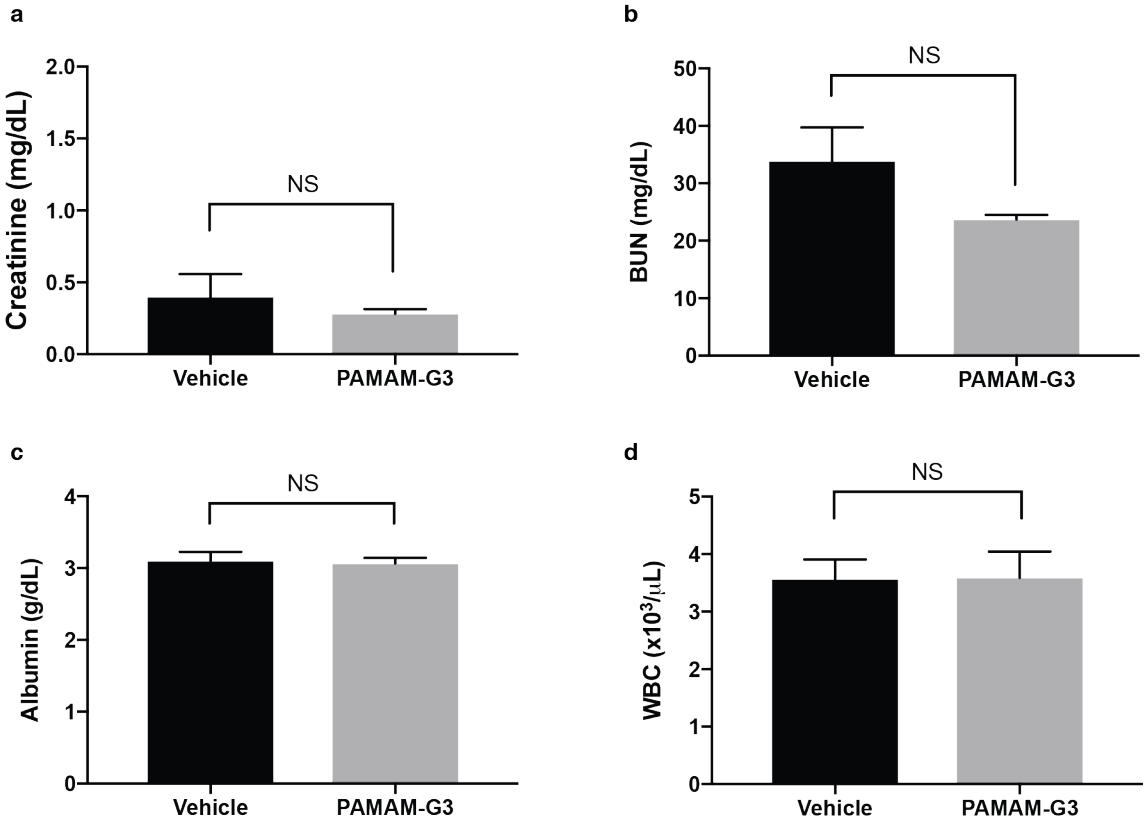

Figure S11. PAMAM-G3 administration in mice is not associated with changes in clinical chemistry values or blood cell counts that indicate systemic toxicity. Healthy, tumor-free C57BL6 mice were injected with PAMAM-G3 (20 mg/kg) (N = 9) or saline vehicle (N = 10) twice weekly for 3 weeks, after which time quantification of plasma creatinine (a), blood urea nitrogen (b), albumin (c), or white blood cell counts (d) was performed. Bar graphs depict mean ± SEM. NS denotes "not significant" by two-tailed t-test.