Bruton's Tyrosine Kinase (BTK)-dependent immune cell crosstalk drives pancreas cancer

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SUPPLEMENTARY METHODS

Immunohistochemistry and Immunofluorescence: Brightfield immunodetection studies using human tissue sections was performed as described previously (1). De-identified human tissue was received from the University of California, San Francisco (UCSF) Department of Pathology under approval from the UCSF Committee on Human Research (05028310), and the Oregon Health and Science University (OHSU) Oregon Pancreas Tumor Registry (OPTR) and courtesy of Dr. Brett Sheppard (institutional review board [IRB] #3609), with patient consent obtained prior to tissue acquisition. The use of samples occurred under ‘‘exempt category 4’’ for individuals receiving de-identified biological specimens. Antibodies used were: αCD45 (H130, 1:500, eBioscience), αCD20 (L26, 1:100, Abcam), αCD3ε (SP7, 1:200, Thermo Scientific), αCD8 (C8/144B, 1:100, Thermo Scientific).

IHC was conducted with FFPE (5 μm) tissue sections of human PDAC subjected to heat-mediated antigen retrieval immersed in citrate buffer (pH 6.0) for 15 min. prior to blocking with 5% goat serum for 30 min. For human IHC, primary murine or rabbit antibodies were then serially stained for 2 hours at RT or O/N at 4º C using mouse anti-CD20 (L-26, 1:100, Santa Cruz), mouse anti-CD8 (C8/144B, 1:100; Thermo Scientific), Rabbit anti-CD3 (SP7, 1:200, Thermo Scientific), mouse anti-CD45 (30-F11, 1:100, eBioscience), rabbit anti-CD11b (1:1000, Abcam), , and rabbit anti-BTK (D3H5, 1:100, Cell Signaling). Following washing of each antibody, primary antibodies were stained with either an anti-mouse or anti-rabbit Histofine Simple Stain MAX PO HRP conjugated polymer (Nichirei Biosciences Inc.) for 30 min. at RT followed by DAB (singleplex) or AEC (multiplex) for peroxidase detection. Slides were scanned at Aperio ImageScope AT (Leica Biosystems) at either 20x (multiplex) or 40x (singleplex) magnification. Samples chosen for multiplex staining were destained in an alcohol gradient following the whole slide scanning and image processing according to a protocol of Glass et. al., (2). Slides were restained sequentially with the indicated antibodies following the antibody stripping protocol in heat retrieval as reported (3). Multiplex images were coregistered using CellProfiler software (Broad Institute), deconvoluted using Image J, pseudocolored and merged in ImageScope (Aperio, Leica). High magnification images were created with a 5x zoom from a 20x original magnification. For murine IHC, immunodetection of murine tissue sections was performed as previously described (4). Antibodies used were as follows: αB220 (RA3-6B2, 1:500, BD Biosciences), αBrdU (MCA2060, 1:2000 AbD Serotec), αCC3 (D175, 1:500, Cell Signaling), αCD11b (M1/70, 1:200, BD Biosciences), αCD8a (53-6.7, 1:50, BD Biosciences), αCD31 (MEC13.3, 1:500, BD Biosciences), αGranzyme B (NB100-684, 1:100, Novus Biologicals), and αSMA (ab5694, 1:250, Abcam). Analysis of positive staining was achieved utilizing Aperio automated scanning (Leica) and IHC quantitation software (ScanScope).

Flow cytometry: Single cell suspensions from blood, spleen, and peritoneal cavity were prepared as previously described (4). Mouse and human tumor tissue was collected and stored in PBS/0.1% soybean trypsin inhibitor prior to enzymatic

dissociation. Mice were cardiac-perfused with 10-15 ml PBS/10 u/ml heparin prior to collection to clear peripheral blood. Samples were finely minced with a scissors and mouse tissue was transferred into DMEM containing 1.0 mg/ml collagenase IV (Gibco), 0.1% soybean trypsin inhibitor, and 50 U/ml DNase (Roche) and incubated at 37°C for 30 min. with constant stirring while human tissue was digested in 2.0 mg/ml collagenase IV, 1.0 mg/ml hyaluronidase, 0.1% soybean trypsin inhibitor, and 50 U/ml DNase for 45 minutes. Suspensions were filtered through a 100 micron filter and washed with FACS buffer (PBS/0.5% BSA/2.0 mM EDTA) prior to staining. Two million total cells were stained with antibodies as indicated. Intracellular detection of pBTK (Y223) and cytokines was achieved following permeabilization with BD Perm Buffer III (BD Biosciences) and eBioscience Fix/Perm respectively. Intracellular detection of cytokines occurred following *ex vivo* stimulation of cells in complete RPMI media containing 50 ng/ml PMA, 500 ng/ml Ionomycin, 2.0 μM Monensin (BD Biosciences) and 1.0 μg/ml LPS. Following staining, samples were acquired on a BD Fortessa or LSRII (BD Biosciences) and analyzed using FlowJo (Treestar) software. Antibodies used for murine FACS were as follows: CD4 (RM4-5, 1:400, Biolegend), CD8 (53-6.7, 1:400, Biolegend), CD19 (6D5, 1:400, Biolegend), B220 (RA3-6B2, 1:400, Biolegend), MHCII (M5/114.15.2, 1:1000, eBioscience), Gr-1 (RB6-8C5, 1:400, eBioscience), Ly6G (1A8, 1:400, eBioscience), Ly6C (HK1.4, 1:400, Biolegend), F4/80 (BM8, 1:400, Biolegend), CD11c (N418, 1:400, eBioscience), CD3 (17A2, 1:200, Biolegend), IL-10 (JES5-16E3, 1:200, eBioscience), CD5 (53-7.3, 1:400, eBioscience), CD1d (1B1, 1:400, BD Biosciences), CD45 (30-F11, 1:4000, eBioscience), Granzyme B (NGZB, 1:200, eBioscience), CD64 (X54-5/7.1, 1:200, Biolegend), CD16/CD32 (93, 1:200, Biolegend), Foxp3 (FJK-16s, 1:400, eBioscience), and pBTK-Y223 (N35-86, 1:400, BD Biosciences), EOMES (Dan11mag, 1:400, eBioscience), PD-1 (RMP1-30, 1:400, Biolegend), CD107a (1D4B, 1:200, Biolegend), IFN-γ (XMG1.2, 1:400, eBioscience), CD138 (281-2, 1:400, BD Bioscience), CD23 (B3B4, 1:800, eBioscience), and IgM (II/41, 1:400, eBioscience). Antibodies used for human FACS were as follows: CD45 (2D1, 1:100, BD Biosciences), CD4 (1:50, OKT4, Biolegend), CD8 (RPA-T8, 1:100, Biolegend), CD19 (HIB19, 1:100, BD Biosciences), HLA-DR (L243, 1:100, eBioscience), CD11c (3.9, 1:200, eBiosciences), CD11b (ICRF44, 1:200, Biolegend), CD15 (HI98, 1:200, Biolegend), CD14 (HCD14, 1:50, Biolegend), CD206 (19.2, 1:100, BD Biosciences), FoxP3 (236A/E7, 1:50, eBioscience), CD3 (OKT3, 1:50, eBioscience), FcεR1α (AEP–37, 1:50, eBioscience), and CD117 (YB5.B8, 1:50, BD Biosciences). CD64 (X54-5/7.1, 1:100, BD Biosciences), and CD16 (3G8, 1:100, Biolegend).

Real-time PCR: Mice were cardiac-perfused with PBS to clear peripheral blood. Following enzymatic digestion as previously described (4), leukocyte subsets were isolated from tissue by FACS using an Influx Cell Sorter or Aria II (BD Biosciences) as previously described (4), and collected directly into RLT lysis buffer for subsequent RNA extraction using RNeasy Micro/Mini kit guidelines (Qiagen). Contaminating DNA was removed with DNase I (Invitrogen). SuperScript III (Invitrogen) was used to reverse transcribe purified RNA into cDNA according to manufacturer's directions. Real-time PCR for gene expression was performed using the TaqMan system (Applied Biosystems) with a 14 cycle preamplification step employed during analysis of FACS-sorted populations. The comparative threshold cycle method was used to calculate fold change in gene expression, which was normalized to *Gapdh* and TATA-binding protein (*Tbp*) as reference genes. For TG100-115 and PCI-32765 tumor studies, qPCR was performed using custom PrimePCR plates (Biorad), where mRNA levels were normalized to *gapdh* as reference gene.

Splenocyte stimulation: Splenic single cell suspensions were harvested from tumor naïve FVB/n male mice and subjected to *in vitro* stimulation with 10 μg/ml αIgM F(ab)'2 fragment for 2 minutes or 25 μg/ml αCD16/CD32 for 10 minutes on ice followed by F(ab)'2 fragment goat anti-rat IgG Fc specific (Jackson Immunolaboratories) for 5 minutes in serum free RPMI media. Following stimulation, cells were washed with PBS/0.5% BSA/2.0 mM EDTA supplemented with phosphatase inhibitors and FACS stained for Live/Dead Aqua (Invitrogen), CD3-APC, CD19-Brilliant Violet 650, CD11b-FITC and MHCII-EFluor450 for 30 min. Cells were then fixed with BD Cytofix (BD Biosciences) for 20 minutes. Fixative was washed off with PBS and cells were permeabilized with BD Perm Buffer III for 30 minutes on ice. Upon removal of permeabilization buffer, cells with stained with anti-pBTK (Y223)-PE for 30 minutes and acquired on a BD Fortessa.

Bone Marrow-derived macrophage isolation and gene expression analysis: Bone marrow was harvested by perfusing femurs of 7-12 week old FcR$\gamma^{+/-}$ male mice with DMEM 10% FBS/1.0% Pen/Strep into plastic culture dishes. Suspensions were filtered through a 70-micron mesh, washed 1x and resuspended in RBC lysis buffer to remove contaminating red blood cells. Following wash of RBC lysis, cells were counted and plated in DMEM 10% FBS/1.0% Pen/Strep/10 ng/ml CSF-1 (Peprotech) at a density of 1 x10^5 cells/ml. Following three days in culture, 50% of the medium was exchanged with fresh complete DMEM with 20 ng/ml CSF-1. After 2 days, cells were visualized to confirm morphological presence of macrophages and one well counted and stained for FACS analysis to ensure macrophage differentiation. If necessary, cells were cultured for one or two more days in fresh complete medium to achieve the desired number of cells for study. Alternatively, bone marrow-derived macrophages were generated from C57BL/6, BTK$^{-/-}$ or p110$\gamma^{-/-}$ mice as described above and polarized with IFN-γ (20 ng/ml, Peprotech)/LPS (100 ng/ml, Sigma) for 24 hours or IL-4 (20 ng/ml, Peprotech) for 48 hours. In some studies, macrophages polarized with IL-4 for 24 h were incubated with purified rat anti-mouse CD16/CD32 (BD Pharmingen) at 1.0 μg/10^6 cells for 10 min 4°C and then incubated with goat anti-rat F(ab')2 (25 μg/ml, Jackson ImmunoResearch) for 24 hour. Total RNA was harvested from macrophages using the RNeasy Mini Kit (Qiagen) according to the manufacture's instructions and cDNA was prepared using 1.0 μg RNA with the qScript cDNA Synthesis Kit (Quanta Biosciences). Sybr green based qPCR was performed using murine primers to *Arg1, Il10, Ccl2, Mmp9, Il12p40, Il1b, Il6, Gapdh, Nos2, p110g, p110d, Btk, Plcg2* and *Tnfa* from Qiagen (QuantiTect Primer sets, Qiagen). mRNA levels were normalized to Gapdh levels (dCt = Ct gene of interest – Ct Gapdh) and reported as either relative mRNA expression (ddCt = $2^{(\text{dCt sample} - \text{dCt control})}$) or as fold change (fold change = $\log_2$ (dCt test / dCt control) with n=3.

Immunoblotting: Macrophages, B cells and in vitro cultured p53 2.1.1 or Ink4a 2.2.1 PDAC cells were solubilized in RIPA buffer containing protease and phosphatase inhibitors. Proteins were separated by gradient gel electrophoresis, electrophoretically transferred to PVDF membranes. PVDF membranes were immunoblotted with antibodies from Cell Signaling: α-Btk (C82B8), α-p110γ (4252), α-p110α (4255), δ-p110δ (100-401-862, Rockland), α-pBTK (clone EP420Y, Epitomics), or α-actin (A2103, Sigma-Aldrich).

Cell Proliferation Assay: Macrophages and in vitro cultured p53 2.1.1 or Ink4a 2.2.1 PDAC cells were plated in 96 well plates and treated with dilutions of PCI-32765, TG100-115 (Targegen, Inc., La Jolla, CA) or DMSO (vehicle control) for

72h (n=3). Thiazolyl blue tetrazolium bromide (5.0 mg/ml, Sigma) was incubated with cells and solubilized with DMSO. Absorbance was determined at 560 nm.

Cell adhesion and integrin activation assays

siRNA transfections: $CD11b^+$ cells were purified from BM by anti-CD11b magnetic bead affinity chromatography according to manufacturer's directions (130-049-601, Miltenyi Biotec). Purified $CD11b^+$ cells or primary murine macrophages were transfected with 100 nM of *Btk* (Mm_Btk_1 and Mm_Btk_2) or *Plcγ2* (Mm_Plcg 2_1 and Mm_Plcg2_5) siRNA or with plasmids + pGFPMax using an AMAXA Mouse Macrophage Nucleofection Kit (VAPA-1009, as previously described (5). After transfection, cells were cultured for 48 h in media containing 20% serum prior to evaluation of cell adhesion or gene expression. At least two siRNA/per gene were tested individually for efficient knockdown of protein expression and for inhibition of adhesion or gene expression.

Adhesion assays: 1.0 X 10^5 calcein-AM labeled $CD11b^+$ cells were incubated on HUVEC monolayers or plastic plates coated with 5.0 μg/ml recombinant soluble VCAM-1 (643-VM-050, R&D Systems) in the absence or presence of dilutions of PCI-32765 or TG100-115 or corresponding dilutions of DMSO for 30 minutes at 37ºC with humidity in the presence of DMEM containing 200 ng/ml SDF-1α or IL-1β (R&D Systems) as previously described (5). After washing three times with warmed medium, adherent cells were quantified using a plate fluorimeter (GeniosPro, TECAN).

Ligand (VCAM-1) binding assay: $5x10^5$ murine bone marrow derived CD11b+ cells were incubated with DMEM, 200ng/ml SDF-1α in DMEM, or DMEM containing DMSO or 1.0 μM PCI-32765 together with 1.0 mg/ml mouseVCAM-1/human Fc fusion protein (643-VM-050, R&D Systems) for 3 min. Cells were washed twice and incubated with donkey anti-human-Fcgamma-PE antibody (709-116-098, Jackson Immunoresearch), fixed and analysed by FACS Calibur. Mean fluorescence intensity of treated cells was compared to that of unstimulated cells (basal).

In vitro macrophage B cell co-culture: Bone marrow derived macrophages were plated in the lower chamber of 24 well co-culture chamber plates in the presence of basal or p53 2.1.1 tumor cell conditioned medium. $CD19^+$ cells were purified by magnetic bead isolation from single cell splenic preparations of d14 p53 2.1.1 tumor bearing mice (CD19 MACS Microbeads, Miltenyi Biotech). Cells were washed with PBS and added to the upper chamber of co-culture chambers (12 mm diameter Millicell Cell Culture Insert, Millipore) at a ratio of 3:1 B cells to macrophages respectively and incubated for 24h at 37°C/5% CO_2. For inhibitor treated conditions, $CD19^+$ cells were incubated with 1.0 μM PCI-32165 for 30 min at 37°C/5% CO_2.

In vivo macrophage and B cell adoptive transfer experiment: $CD11b^+Gr1^-$ cells were isolated from single cell suspensions of WT p53 2.1.1 tumors. $CD19^+$ cells were isolated from single cell splenic preparations from d14 tumor bearing mice by magnetic bead isolation. Purified cells were mixed 1:1 for $CD11b^+Gr1^-$ cells or 1:5 for $CD19^+$ cells with p53 2.1.1 tumor cells, and 5 x 10^5 total tumor cells were injected into host mice. For inhibitor treated groups, $CD11b^+Gr1^-$ or $CD19^+$ cells were incubated with 1.0 μM PCI-32765 for 30 min before the addition of tumor cells. Inoculated mice were further treated by intradermal injections of PCI-32765 at 3 and 6 days post inoculation and mice were sacrificed and tumor weights were determined.

Mast cell quantitation: Mast cells were identified by 0.1% Toluidine blue (1% w/v NaCl, pH 2.3) staining of FFPE

tissue sections for 2 min., followed by an alcohol destaining process. Following staining, mast cells were identified by their purple metachromasia and degranulated mast cells were identified by appearance of punctate granules loosely arranged within the cytoplasm and pericellular space. Percent of degranulated mast cells was calculated by dividing the number of degranulated mast cells by the total number of mast cells within in a given tissue section.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1. B cells in human PDAC. A. mRNA expression of *CD20* (*MS4A1*), *IgG1* (*IGHG1*), *IgG3* (*IGHG3*), and *IgM* (*IGHM*) in human PDAC tumors (blue) versus healthy (pink) pancreas analyzed from RNA sequence data compiled from the Oncomine database. Data are represented as box-and-whisker plots depicting median fold change value compared to normal tissue, displaying the first and third quartiles at the end of each box, with the maximum and minimum at the ends of the whiskers. 39 PDAC patients and 39 healthy controls were assessed in each graph. **B.** IgG levels in human plasma from patients with various pancreas neoplasms versus healthy controls as determined via ELISA. Healthy, n = 6; PDAC, n = 13; IPMN, n = 12; pancreatitis, n = 10; PNET, n = 5. Data are represented as box-and-whisker plots depicting median fold change value compared to normal tissue, displaying the first and third quartiles at the end of each box, with the maximum and minimum at the ends of the whiskers. **C.** FACS gating strategy for single cell suspensions from primary human PDAC tissue collected following surgical resection. **D.** Shown are representative FACS quantitation of individual cell populations representing complexity analysis of populations shown in Fig. 1C.

Supplementary Figure S2. B cells and FcRγ-positive cells regulate murine PDAC. A. Photomicrographs of end-stage orthotopic Ink4 2.2, and p53 2.1.1 PDAC tumor sections, as compared to transgenic KIC and KPC PDAC tumors sections stained for B220. **B and C**. Representative photomicrographs of Ink4 2.2 and p53 2.1.1-derived orthotopic PDAC tumors from end-stage mice from syngeneic $JH^{+/-}$, $JH^{-/-}$ (**B**), $FcR\gamma^{+/-}$ and $FcR\gamma^{-/-}$ (**C**) mice. Shown are representative H&E and Gomori trichrome-stained FFPE tissue sections. **D.** FACS gating strategy for single cell suspensions from primary mouse PDAC tissue collected at necropsy. **E.** FACS analysis of B cell subsets day 28 end-stage Ink4 2.2 PDAC tumors following no treatment, PCI-32765 alone, Gem alone, or Gem + PCI-32765. B cell subsets were identified and gated based on the following markers: total B cells: $CD45^+CD19^+CD3^-$, Bregs: $CD5^+CD1d^{hi}$, Plasma Cells: $CD138^+IgM^{lo}$, Plasma Blasts: $CD138^+IgM^{hi}$, B1a: $IgM^{hi}CD23^-CD5^+$, B1b: $IgM^{hi}CD23^-CD5^-CD1d^{lo}$, Marginal Zone (Mz) B: $IgM^{hi}CD23^-CD5^-CD1d^{hi}$, Follicular B: $IgM^{lo}CD23^+CD5^-$. All B cell subsets were gated as a percentage of total $CD19^+$ B cells. Data from two independent experimental studies is shown. Statistical significance was determined using the student's T test with * reflecting p values <0.05. **F.** FACS analysis of single cell suspensions reflecting cell types shown, isolated from Ink4 2.2-derived PDAC (n=5 mice) at end-stage and stained for FcγR1 (CD64) and FcγRII/III (CD16/CD32). Data are background subtracted from MFI of counterpart cells from $FcR\gamma^{-/-}$ tumor bearing mice and reflective of 2 independent experiments. **G.** FACS analysis of single cell suspensions reflecting cell types shown, isolated from Ink4 2.2-derived PDAC grown in $FcR\gamma^{+/-}$ versus $FcR\gamma^{-/-}$ mice at end-stage and stained for FcγR1 (CD64) and FcγRII/III (CD16/CD32). Data are reflective of 2 independent experiments.

Supplementary Figure S3. Leukocytes and BTK in human and murine PDAC. A. Composite photomicrograph showing immunoreactivity for CD45 (light green), CD20 (white), CSF-1R (light blue), CD11b (red), BTK (orange), and DNA (dark blue) in a human PDAC FFPE tissue section. **B.** Phosphorylation (Y223) levels of murine BTK in B cell receptor (BCR)- (αIgM) or FcγR- (αCD16/CD32) stimulated naïve splenocytes as measured by FACS and displayed as mean fluorescent intensity (MFI) between the indicated leukocyte populations. Groups were assayed in triplicate in two independent experiments. **C.** Percent change in growth of Ink4 2.2 PDACs in syngeneic mice following administration

of Gem, αCSF1-neutralizing mAb (clone 5A1), or Gem and αCSF1 as depicted by treatment shown in Fig. 6A. Quantitation of macrophage depletion by FACS of single cell suspensions from PDAC tumors from treatment groups as shown. Data from 2 independent experiments is shown. Each data point reflects an individual tumor with statistical means shown. **D.** Relative mRNA expression of PI3K *isoforms p110α, β, γ, δ* and *Btk* in primary murine macrophages and cultured PDAC clones p53 2.1.1 and Ink4a 2.2. Error bars represent standard errors of the mean. Statistical significance was determined using the student's T test or one-way Anova when analyzing more than two groups, ** $p < 0.01$, *** $p < 0.001$.

Supplementary Figure S4: Macrophage PI3Kγ activates BTK to promote PDAC progression. **A.** Validation of *Btk* siRNA knockdown. **B.** Effect of PCI-32765 on adhesion of SDF-1α-stimulated primary myeloid cells to endothelial monolayers. **C.** Effect of PCI-32765 on adhesion of IFNγ-stimulated primary B cells to endothelial monolayers. Inset, FACS plot showing purity of isolated B cells. **D.** Effect of PLCγ siRNA on SDF-1α and IL-1β-stimulated myeloid cell adhesion to VCAM-1. **E.** Validation of PLCγ siRNA knockdown. **F.** pBTK/BTK Western blotting (cropped gels) in wild type (WT) and $p110\gamma^{-/-}$ IL-4-stimulated primary macrophages. Ratio of pBTK to total BTK is listed under each condition. **G-H.** Effect of PCI-32765 and $p110\gamma^{-/-}$ on IFNγ/LPS (**G**) and IL-4 (**H**) stimulated macrophage gene expression, graphed as mRNA expression relative to basal expression. **I**. Effect of BTK knockdown and embryonic deletion on gene expression of IL-4-polarized murine macrophages. Statistical significance was determined using the student's T test or one-way Anova when analyzing more than two groups, * $p < 0.01$, ** $p < 0.01$, *** $p < 0.001$.

Supplementary Figure S5. BTK and PI3Kγ-inhibition decrease PDAC growth. **A.** FACS plot showing purity of isolated $Gr1^{-}CD11b^{+}$ cells and B cells used in Figure 5A. **B-C**. Effect of PCI-32765 and vehicle control on (**B**) bone marrow-derived macrophage (BMM) and (**C**) p53 2.1.1 PDAC tumor cell viability. **D.** Effect of TG100-115 and vehicle control on p53 2.1.1 PDAC tumor cell viability. **E**. CBC analysis of murine peripheral blood harvested 2 days post-treatment or 10 days post-treatment with PCI-32765 +/- Gem. Total white blood cells (WBC), polymorphonuclear leukocytes (PMN) and lymphocytes (lympho) are shown from one representative experiment. N = 8 mice/experimental group. **F.** Representative FACs plots of $CD19^{+}CD220^{+}$ B cells from p53 2.1.1 tumors grown in WT or $p110\gamma^{-/-}$ animals that were treated with PCI-32765 or vehicle. **G**. Representative photomicrographs showing immunofluorescent staining (green) of $CD11b^{+}$ myeloid cells in mice bearing p53 2.1.1 tumors treated with vehicle (Control), PCI-32765 (PCI), TG100-115 (TG100), gemcitabine (Gem) or combinations thereof. 'Control' indicates vehicle-treated tumor-bearing mice. 'Normal' indicates tumor-naïve pancreata. Scale bar: 100 μm. **H**. mRNA expression of Arginase 1 in tumor-derived myeloid cells and whole tumors from WT or $p110\gamma^{-/-}$ animals treated with PCI-32765 or vehicle (n=5). Error bars represent standard error of the mean. Statistical significance was determined using the student's T test or one-way Anova when analyzing more than two groups, *** $p < 0.001$.

Supplementary Figure S6: BTK inhibition improves gemcitabine response in late-stage PDAC. A. Representative photomicrographs of tissue sections from Ink4 2.2-derived PDAC tumors from mice treated as indicated (on top), e.g., vehicle control, PCI-32765, Gem, PCI-32765/Gem, and stained via immunodetection of CD45, Gomori trichrome, and granzyme B. **B.** FACS analysis of Ink4 2.2 PDAC tumors harvested at day 23 post-implantation and assessed for $CD8^+$ T cell effector/memory markers PD-1 and EOMES. Data is displayed as a percentage of $CD3^+CD8^+$ cells. **C.** FACS analysis reflecting single cell suspensions of Ink4 2.2-derived tumors from mice treated with depleting CD8 antibodies as indicated (Fig. 6A). **D.** Toluidine blue staining of Ink 2.2-derived PDAC tumors to reveal mast cells, with higher magnification images revealing degranulation (right). Graphs indicate quantitation of total mast cells (left) and percentage of mast cells evidencing a degranulation phenotype as indicated by pericellular granules in the treatment groups shown. Error bars represent standard error of the mean. Statistical significance was determined using the student's T test or one-way Anova when analyzing more than two groups, ns: not statistically significant.

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