

# Bruton Tyrosine Kinase–Dependent Immune Cell Cross-talk Drives Pancreas Cancer

Andrew J. Gunderson<sup>1</sup>, Megan M. Kaneda<sup>2</sup>, Takahiro Tsujikawa<sup>1,3</sup>, Abraham V. Nguyen<sup>2</sup>, Nesrine I. Affara<sup>4</sup>, Brian Ruffell<sup>1</sup>, Sara Gorjestani<sup>2</sup>, Shannon M. Liudahl<sup>1</sup>, Morgan Truitt<sup>5</sup>, Peter Olson<sup>5</sup>, Grace Kim<sup>4,6</sup>, Douglas Hanahan<sup>7</sup>, Margaret A. Tempero<sup>6,8</sup>, Brett Sheppard<sup>9,10</sup>, Bryan Irving<sup>11</sup>, Betty Y. Chang<sup>12</sup>, Judith A. Varner<sup>2,13</sup>, and Lisa M. Coussens<sup>1,10</sup>

## ABSTRACT

Pancreas ductal adenocarcinoma (PDAC) has one of the worst 5-year survival rates of all solid tumors, and thus new treatment strategies are urgently needed. Here, we report that targeting Bruton tyrosine kinase (BTK), a key B-cell and macrophage kinase, restores T cell–dependent antitumor immune responses, thereby inhibiting PDAC growth and improving responsiveness to standard-of-care chemotherapy. We report that PDAC tumor growth depends on cross-talk between B cells and FcR $\gamma$  tumor-associated macrophages, resulting in T<sub>H</sub>2-type macrophage programming via BTK activation in a PI3K $\gamma$ -dependent manner. Treatment of PDAC-bearing mice with the BTK inhibitor PCI32765 (ibrutinib) or by PI3K $\gamma$  inhibition reprogrammed macrophages toward a T<sub>H</sub>1 phenotype that fostered CD8<sup>+</sup> T-cell cytotoxicity, and suppressed PDAC growth, indicating that BTK signaling mediates PDAC immunosuppression. These data indicate that pharmacologic inhibition of BTK in PDAC can reactivate adaptive immune responses, presenting a new therapeutic modality for this devastating tumor type.

**SIGNIFICANCE:** We report that BTK regulates B-cell and macrophage-mediated T-cell suppression in pancreas adenocarcinomas. Inhibition of BTK with the FDA-approved inhibitor ibrutinib restores T cell–dependent antitumor immune responses to inhibit PDAC growth and improves responsiveness to chemotherapy, presenting a new therapeutic modality for pancreas cancer. *Cancer Discov*; 6(3); 270–85. ©2015 AACR.

See related commentary by Roghanian et al., p. 230.

See related article by Pylayeva-Gupta et al., p. 247.

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<sup>1</sup>Department of Cell, Developmental and Cancer Biology, Oregon Health and Science University, Portland, Oregon. <sup>2</sup>Moore's Cancer Center, University of California, San Diego, La Jolla, California. <sup>3</sup>Department of Otolaryngology-Head and Neck Surgery, Oregon Health and Science University, Portland, Oregon. <sup>4</sup>Department of Pathology, University of California, San Francisco, California. <sup>5</sup>Department of Biochemistry and Biophysics, University of California, San Francisco, California. <sup>6</sup>Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, California. <sup>7</sup>Swiss Institute for Experimental Cancer Research, Swiss Federal Institute of Technology, Lausanne, Switzerland. <sup>8</sup>Department of Medicine, University of California, San Francisco, California. <sup>9</sup>Department of Surgery, Oregon Health and Science University, Portland, Oregon. <sup>10</sup>Knight Cancer Institute, Oregon Health and Science University, Portland, Oregon. <sup>11</sup>Genentech, South San Francisco, California. <sup>12</sup>Pharmacyclics Inc., Sunnyvale, California. <sup>13</sup>Department of Pathology, University of California, San Diego, La Jolla, California.

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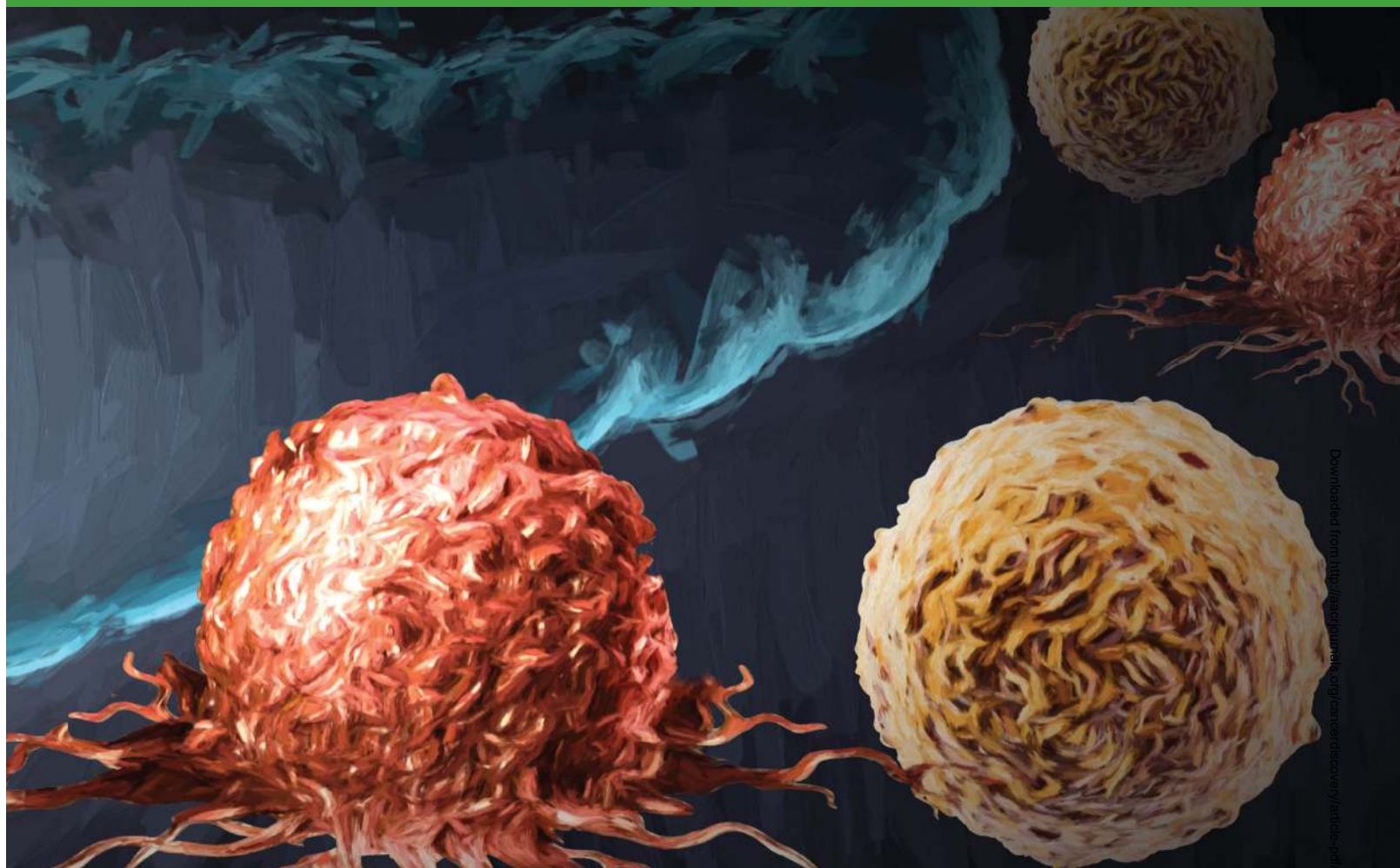
A.J. Gunderson, M.M. Kaneda, J.A. Varner, and L.M. Coussens contributed equally to this article.

Current address for B. Irving: CytomX Therapeutics Inc., South San Francisco, CA.

**Corresponding Authors:** Lisa M. Coussens, Cell, Developmental and Cancer Biology, Knight Cancer Institute, Oregon Health and Science University, 181 SW Sam Jackson Park Road, Code 1215, Portland, OR 97239-3098. Phone: 503-494-7811; Fax: 503-494-4253; E-mail: coussensl@ohsu.edu; and Judith A. Varner, Department of Pathology, Moore's Cancer Center, University of California, San Diego, 3855 Health Sciences Drive, La Jolla, CA 92093-0819. Phone: 858-822-0086; Fax: 858-822-1325; E-mail: jvarner@ucsd.edu

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## INTRODUCTION

The contribution of tissue-resident and recruited leukocytes to the progression of solid tumors is now a widely accepted mechanism of pathogenesis (1, 2), and one that is gaining notable traction in the clinic (3). Although many studies have dissected the numerous activities leukocytes exert toward regulating neoplastic progression, tractable immune-based targets for anticancer therapy are only just emerging.

Ductal adenocarcinoma of the pancreas (PDAC) is a devastating disease with one of the lowest 5-year survival rates of all solid tumors (4). Currently, 7.2% of patients with newly diagnosed pancreatic cancer will survive 5 years following diagnosis, due in part to the fact that PDAC is rarely detected at an early stage; instead, the majority of patients present with locally unresectable or metastatic disease (5). Because standard therapies have only a modest impact on survival (6, 7), novel therapeutic and diagnostic strategies are urgently needed.

A characteristic feature of PDAC tumors is the presence of abundant infiltrating leukocytes representing both lymphoid and myeloid lineages (8); thus, we sought to identify functionally significant immune-based programs regulating pancreatic carcinogenesis that would be tractable for therapeutic

intervention. We report here that B cell–macrophage interactions promote PI3K $\gamma$ - and Bruton tyrosine kinase (BTK)-dependent macrophage T<sub>H</sub>2 polarization, leading to immune suppression and PDAC progression. Using mouse models of PDAC, targeted inhibition of BTK, a common signaling molecule in B cells and myeloid cells, resulted in slowed PDAC tumor growth, abated immune suppression, impeded late-stage tumor growth, and improved responsiveness to standard-of-care chemotherapy (CTX), indicating that targeting BTK therapeutically could provide long-term antitumor control for this devastating malignancy.

## RESULTS

### B Cells and FcR $\gamma$ -Positive Cells in Human PDAC

We previously identified a protumorigenic role for CD20<sup>+</sup> B cells in solid tumors using murine models of squamous cell carcinogenesis, and demonstrated that therapeutic depletion of B cells in squamous cell carcinoma (SCC)-bearing mice resulted in an improved response to CTX by CD8<sup>+</sup> T cell-dependent mechanisms (9). To identify which human solid tumors might be regulated by protumorigenic B cells, we examined cDNA microarrays of ~3,000 human tumors to assess levels of *CD20* and immunoglobulin (*Ig*) mRNA expression



relative to corresponding normal tissue (9). As expected, human SCCs of the vulva and head and neck exhibited high expression of both mRNAs (9). Human PDACs also exhibited increased *CD20* and *Ig* expression relative to corresponding healthy pancreas tissue, whereas intrapapillary mucinous neoplasias (IPMN) and islet cell carcinomas did not (Fig. 1A). Using an independent data set, we confirmed increased expression of *CD20*, *IgG3*, and *IgM* mRNA in human PDACs (Supplementary Fig. S1A) and correlated with significantly increased plasma IgG in patients with late-stage PDAC (Supplementary Fig. S1B). To quantitatively evaluate the presence of specific leukocyte lineages in healthy pancreata versus regions of resected PDACs, we evaluated fresh single-cell suspensions from surgically resected healthy pancreata and primary human PDAC tumors by polychromatic flow cytometry (FACS; Supplementary Fig. S1C). We found that CD45<sup>+</sup> leukocyte infiltration of PDAC tumors was significantly increased as compared with healthy pancreas tissue (Fig. 1B), and in PDAC from either chemo-naïve or chemo-treated patients, tumors were dominated by B cells and CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Fig. 1C; Supplementary Fig. S1D), similar to reports from other studies (8).

Based on our previous data indicating that B cells regulate protumorigenic programming of Ig receptor gamma (FcγR)-positive myeloid cells (9), we next evaluated publicly available data sets for FcγR expression. We found that *FCGR1*, *FCGR2B*, and *FCGR3B* mRNAs were increased in PDACs as compared with healthy pancreas (Fig. 1D). In addition, we evaluated the frequency of leukocytes expressing CD64 (FcγR1) and CD16 (FcγRIII), the activating forms of FcγR, in human PDAC tumors, and found the highest levels of CD64 on macrophages, dendritic cells (DC), and immature monocytes, and the highest levels of CD16 instead on eosinophils and neutrophils (Fig. 1E). Based on these collective data, we hypothesized that, similar to murine SCCs, B cells cooperate with FcγR-positive myeloid cells to foster PDAC tumorigenesis.

### B Cells and FcγR-Positive Myeloid Cells Foster PDAC Tumorigenesis

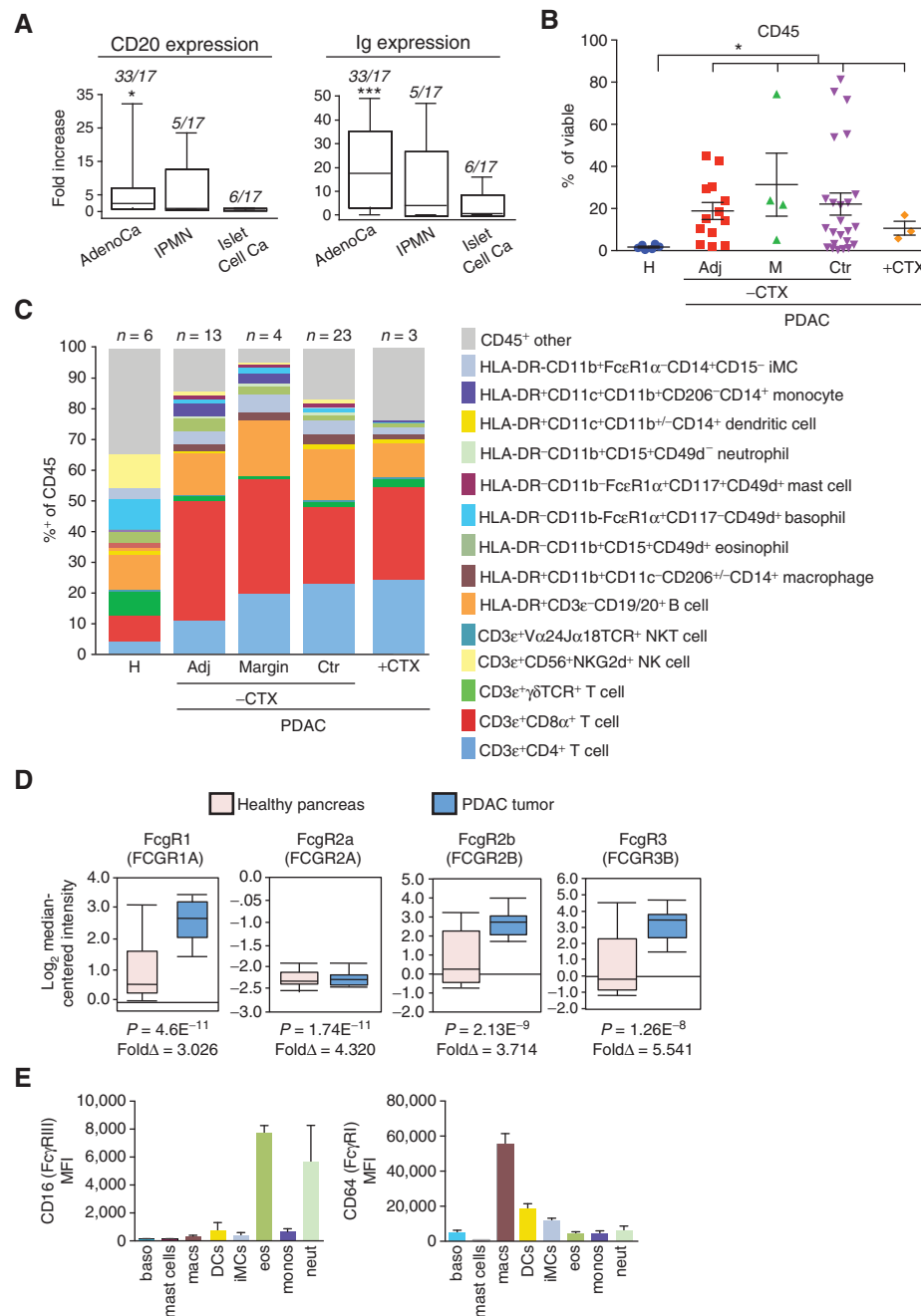
To test the hypothesis that B cells collaborate with myeloid cells to promote PDAC tumorigenesis, we investigated tumor growth of two syngeneic murine PDAC cell lines derived from primary pancreatic carcinomas of transgenic *Pdx-Cre; LSL-Kras<sup>G12D</sup>* mice (10–12) harboring null mutations in *p16<sup>Ink4a</sup>* (Ink4 2.2) or *Trp53* (p53 2.1.1; refs. 13–16). Both cell lines generated PDACs that were histologically similar to PDACs from the transgenic mice from which they were derived (Supplementary Fig. S2A–S2C). PDAC tumors derived from both cell lines also exhibited similar infiltration by CD45<sup>+</sup> leukocytes as a percentage of viable cells in tumors (Fig. 2A; Supplementary Fig. S2D), as well as significant B-cell infiltration as compared with wild-type (WT) pancreas tissue as revealed by FACS analysis, predominated by IgM<sup>hi</sup>CD23<sup>+</sup> transitional 2 cells, IgM<sup>hi</sup>CD23<sup>+</sup>CD5<sup>+</sup> B1a cells, IgM<sup>hi</sup>CD23<sup>+</sup>CD5<sup>+</sup>CD1d<sup>lo</sup> B1b cells, IgM<sup>lo</sup>CD23<sup>+</sup>CD5<sup>+</sup> follicular B cells, IgM<sup>lo</sup>CD23<sup>+</sup> memory B cells, and, to a lesser extent, B cells reflecting marginal zone, regulatory, plasma blast, and plasma cells (Fig. 2B; Supplementary Fig. S2E), and/or immunohistochemical analysis (Supplementary Fig. S2A).

To determine if B cells or FcγR-positive myeloid cells imparted a growth advantage to orthotopic PDACs, cell lines were implanted into syngeneic B cell-proficient (JH<sup>+/+</sup> or <sup>+/+</sup>) or B cell-deficient JH<sup>-/-</sup> mice (Fig. 2B and C). JH<sup>-/-</sup> mice possess a deletion in the J segment of the Ig heavy-chain locus and thus do not express IgM or IgG, and thus have no mature B cells in bone marrow or periphery due to blocked B-cell differentiation at the large, CD43<sup>+</sup> precursor stage (17). Tumor cells were also implanted into Ig receptor FcγR-proficient (FcγR<sup>+/+</sup> or <sup>+/+</sup>) and FcγR-deficient (<sup>-/-</sup>) mice (18), and tumor growth kinetics and characteristics were evaluated longitudinally (Fig. 2C and D; Supplementary Fig. S2B and S2C). PDACs grown in either B cell-deficient or FcγR-deficient mice were significantly smaller than littermate controls (Fig. 2C and D) and exhibited significantly reduced desmoplasia, as determined by alpha smooth muscle actin (αSMA) immunoreactivity (Fig. 2C and D) and Gomori trichrome staining (Supplementary Fig. S2B and S2C).

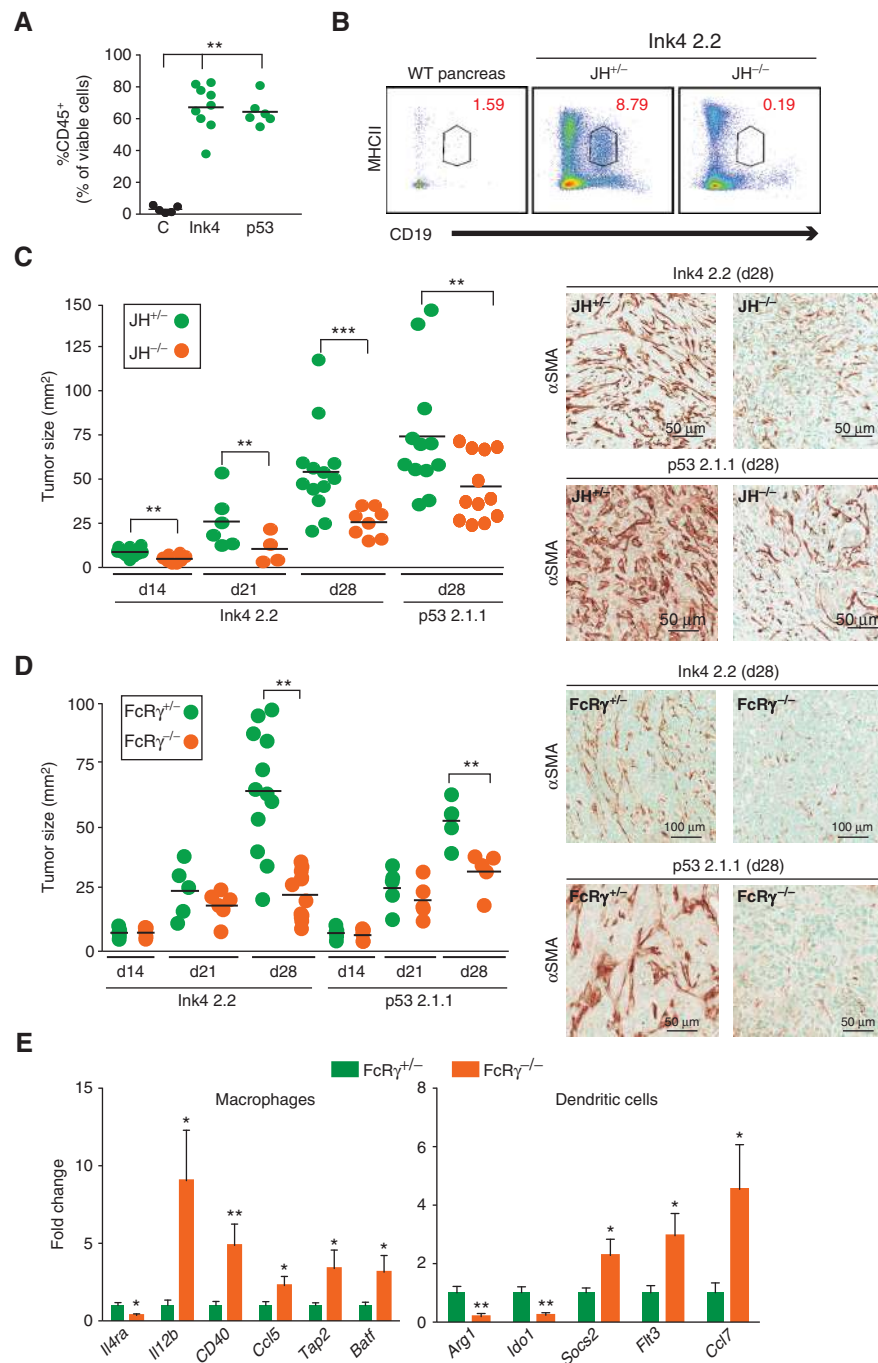
Similar to human PDAC expression of *FCGR1*, *FCGR2B*, and *FCGR3B* mRNAs (Fig. 1D), we evaluated expression of CD64 FcγRI and CD16/CD32 FcγRII/III by FACS (Supplementary Fig. S2F) and found high-level expression on tumor-infiltrating macrophages and DCs, and to a lesser degree monocytes and neutrophils (Supplementary Fig. S2F). Macrophages, DCs, monocytes, and neutrophils infiltrating Ink4 2.2-derived PDAC tumors grown in FcγR-deficient (*Il4ra*) mice exhibited no expression of either FcγRI or FcγRII/III molecules (Supplementary Fig. S2G). Because we previously reported that FcγR<sup>-/-</sup> macrophages in SCC tumors have a shifted gene expression profile favoring expression of mRNAs characterizing T<sub>H</sub>1 phenotypes (9, 19), we isolated CD45<sup>+</sup>CD11b<sup>+</sup>MHCII<sup>+</sup>F4/80<sup>+</sup>Ly6C<sup>-</sup> macrophages and CD45<sup>+</sup>CD11b<sup>+</sup>MHCII<sup>+</sup>F4/80<sup>+</sup>CD11c<sup>+</sup> DCs from Ink4 2.2-derived PDAC tumors harvested from FcγR-proficient versus FcγR-deficient mice, and evaluated a panel of characteristic T<sub>H</sub>1 (*Il12b*, *Cd40*, *Ccl5*, *Tap2*, *Batf*, *Flt3*, *Ccl7*, *Socs2*) and T<sub>H</sub>2 (*Il4ra*, *Arg1*, *Ido1*) mRNAs. We found significant skewing of both cell types toward a T<sub>H</sub>1 phenotype in FcγR-deficient mice (Fig. 2E). These data, together with diminished PDAC growth observed in B cell-null JH<sup>-/-</sup> mice and Ig receptor-null FcγR<sup>-/-</sup> mice, thus supported the hypothesis that B cells, as well as subsets of myeloid cells, influence PDAC tumor growth.

### B Cell and Myeloid Cell Signaling Pathways in PDAC

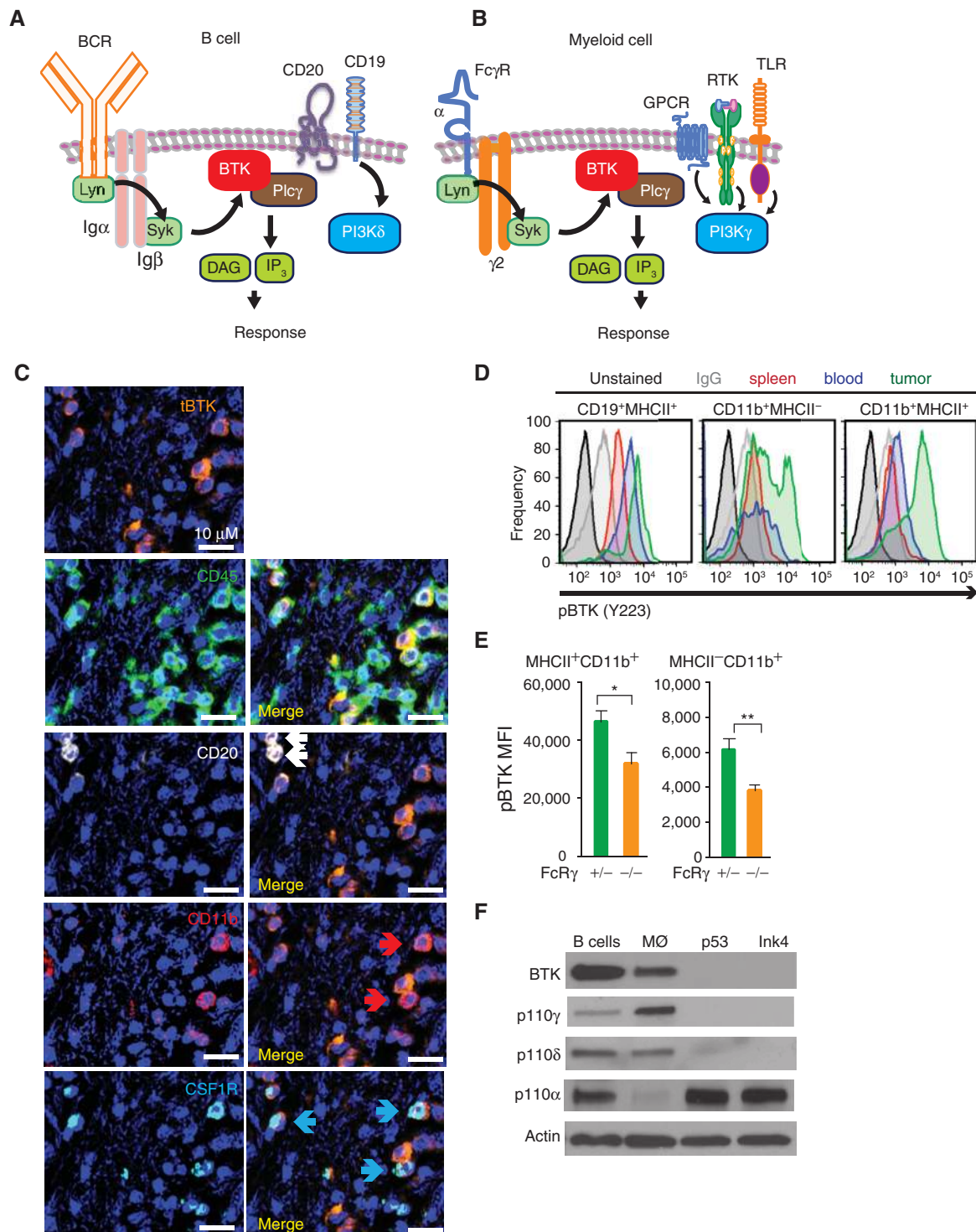
Based on these findings and our previous data indicating that B cells and tumor-associated macrophages impede cytotoxic T-cell (CTL) activity to regulate solid tumor development (19, 20), we hypothesized that inhibitors of common signaling pathways active in both B cells and infiltrating macrophages, such as BTK or PI3K signaling, might be efficacious against PDAC tumors (Fig. 3A and B). BTK is a member of the TEC family of cytoplasmic protein tyrosine kinases expressed by multiple hematopoietic-lineage cells; in B lymphocytes, BTK is activated by the B cell receptor (BCR) pathway, whereas in macrophages, BTK is activated downstream of FcγR by spleen tyrosine kinase (Syk; refs. 21, 22). Thus, we examined human PDAC specimens for the presence of BTK<sup>+</sup> cells in tissue sections and found BTK immunoreactivity in CD45<sup>+</sup>



**Figure 1.** Leukocytes in human PDAC. **A**, relative CD20 and Ig mRNA expression in human pancreatic ductal adenocarcinoma (AdenoCa;  $n = 33$ ), IPMN ( $n = 5$ ), and islet cell carcinomas (Islet Cell Ca;  $n = 6$ ), as compared with healthy pancreas tissue ( $n = 17$ ) assessed by Affymatrix Human U133 Plus 2 microarrays. Data are represented as box-and-whisker plots depicting median fold-change value compared with 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. Statistical significance was determined via the Wilcoxon rank-sum test with \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ . **B**, FACS quantification of CD45<sup>+</sup> cells as a percentage of viable cells from pancreas tissues reflecting healthy (H) pancreata ( $n = 6$ ), pancreas tissue adjacent to PDAC (Adj;  $n = 13$ ), PDAC tumor margins (M;  $n = 4$ ) and tumor centers (Ctr;  $n = 23$ ) from patients who had not received prior chemotherapy (-CTX), and PDACs ( $n = 3$ ) from patients treated with standard-of-care CTX (+CTX). Each data point reflects an individual piece of tissue. **C**, leukocyte complexity of healthy human pancreas ( $n = 6$ ), and pancreas tissue isolated from patients with PDAC evaluated by polychromatic flow cytometry of single-cell suspensions and evaluated for expression of the lineage markers shown. Data represent mean leukocyte complexity for leukocyte lineages shown (right) in tumor tissue resected from healthy pancreata (H;  $n = 6$ ), pancreata tissue adjacent to PDAC (Adj;  $n = 13$ ), tissue resected from PDAC margins ( $n = 4$ ) or tumor centers (Ctr;  $n = 23$ ), and PDACs ( $n = 3$ ) from patients treated with standard-of-care CTX (+CTX). Data reflecting individual populations is provided in Supplementary Fig. S1D. **D**, mRNA expression of FcγR isoforms from humans with healthy pancreas versus patients with PDAC tumors. Data were compiled from gene expression available in Oncomine (<https://www.oncomine.org>). P values and fold change in gene expression are shown. **E**, FACS histogram showing relative frequency of CD64 (FcγR1) and CD16 (FcγRIII) expression on leukocytes infiltrating human PDAC. Data shown are reflective of 20 human PDACs. Lineage markers for cell types shown are as indicated in **C** and reflect basophils (baso), mast cells, macrophages (macs), DCs, inflammatory monocytes (iMCs), eosinophils (eos), monocytes (monos), and neutrophils (neut). Adjacent (Adj) normal indicates tissue isolated from pancreas tissue proximal to resected PDAC tumors.



**Figure 2.** Orthotopic PDAC growth is regulated by B cells and FcR $\gamma$ -positive myeloid cells. **A**, murine PDAC cell lines, derived from *Pdx-Cre; LSL-Kras<sup>G12D</sup>* mice harboring an *Ink4a/Arf<sup>fllox/+</sup>* allele (Ink4 2.2) or a *p53<sup>fllox/+</sup>* allele (p53 2.1.1), were injected orthotopically into the pancreas, and subsequent tumors evaluated by FACS for CD45-positive cellular infiltrates. Graph shows the percentage of CD45<sup>+</sup> cells as a percentage of viable cells. **B**, representative CD19<sup>+</sup>MHCII<sup>+</sup> FACS plot gated on viable CD45<sup>+</sup> cells from tumor-naïve pancreas and Ink4 2.2-implanted PDAC tumors in syngeneic JH<sup>+/-</sup> and JH<sup>-/-</sup> mice evaluated 28 days after implantation. **C**, Ink4 2.2 and p53 2.1.1-derived PDACs were quantitatively evaluated in syngeneic JH<sup>+/-</sup> and JH<sup>-/-</sup> mice for tumor area in serial H&E-stained tissue sections reflecting tumors isolated on days 14, 21, and 28 after implantation. On right, representative photomicrographs reflecting immunodetection of  $\alpha$ SMA in end-stage Ink4 2.2 and p53 2.1.1-derived orthotopic PDAC tumors. **D**, Ink4 2.2 and p53 2.1.1-derived PDACs were quantitatively evaluated in syngeneic FcR $\gamma$ <sup>+/-</sup> and FcR $\gamma$ <sup>-/-</sup> mice for tumor area in serial H&E-stained tissue sections reflecting tumors isolated on days 14, 21, and 28 after implantation. Right, representative photomicrographs reflecting immunodetection of  $\alpha$ SMA in end-stage Ink4 2.2 and p53 2.1.1-derived orthotopic PDAC tumors. **E**, qRT-PCR analysis from cDNA reflecting FACS-purified macrophages (M $\phi$  = CD45<sup>+</sup>CD11b<sup>+</sup>MHCII<sup>+</sup>F4/80<sup>+</sup>Ly6C<sup>-</sup>) or DC (CD45<sup>+</sup>CD11b<sup>+</sup>MHCII<sup>+</sup>F4/80<sup>-</sup>CD11c<sup>+</sup>) from Ink4 2.2 PDAC-derived tumors harvested from day 21 FcR $\gamma$ <sup>+/-</sup> ( $n = 10$ ) or FcR $\gamma$ <sup>-/-</sup> ( $n = 8$ ) mice. Displayed are fold changes in genes that reached statistically significant differences. Data are compiled from two independent experiments. For graphs in **A**, **C**–**E**, statistical means are shown, with  $P$  values determined by either Student  $t$  test or one-way ANOVA when analyzing more than two groups, with \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . For graphs in **C** and **D**, each data point reflects mean tumor size from one mouse based on quantitative morphometry of 5 FFPE sections, resulting from 3 independent experiments.



**Figure 3.** BTK and PI3K in PDAC-infiltrating B cells and myeloid cells. **A** and **B**, cartoons depicting BCR- and FcR $\gamma$ -activated BTK signaling in B cells and myeloid cells. **C**, representative photomicrograph showing immunodetection of BTK, CD45, CD20, CD11b, and CSF1R in a human PDAC FFPE section. Arrows in “merged” images indicate double-positive cells. Magnification is shown. **D**, intracellular FACS detection of activated phospho-BTK (pBTK; Y223) in single cells harvested from peripheral blood, spleen, and tumor tissue of Ink4 2.2-implanted syngeneic mice, gated on CD19<sup>+</sup>MHCII<sup>+</sup> B cells, and CD11b<sup>+</sup>MHCII<sup>-</sup> and CD11b<sup>+</sup>MHCII<sup>+</sup> myeloid cells. Also shown are unstained and IgG1 isotype control stained cells. Representative data from one experiment ( $n = 7$  mice) reflective of 2 independent experiments are shown. **E**, intracellular FACS detection of pBTK (Y223) in MHCII<sup>+</sup>CD11b<sup>+</sup> and MHCII<sup>-</sup>CD11b<sup>+</sup> cells isolated from end-stage Ink4 2.2 PDAC tumors from syngeneic FcR $\gamma$ <sup>+/-</sup> or FcR $\gamma$ <sup>-/-</sup> mice. Data from one representative experiment ( $n = 8$  mice per experimental group) are shown and are reflective of 2 independent experiments. **F**, cropped Western blot images showing expression of BTK, PI3K $\gamma$  (p110 $\gamma$ ), PI3K $\delta$  (p110 $\delta$ ), PI3K $\alpha$  (p110 $\alpha$ ), and actin in murine PDAC-derived B cells, primary murine macrophages (MØ), and cultured PDAC clones p53 2.1.1 (p53), and Ink4a 2.2 (Ink4).



leukocytes, CD20<sup>+</sup> B cells, and CD11b<sup>+</sup> and colony-stimulating factor 1 receptor (CSF1R)-positive myeloid cells (Fig. 3C; Supplementary Fig. S3A). In murine PDAC tumors, we identified activated BTK (pBTK) cells in single-cell suspensions, most prominently in tumor-resident CD19<sup>+</sup>MHCII<sup>+</sup> B cells and CD11b<sup>+</sup> myeloid cells (Fig. 3D). Relative to unstimulated cells, the mean fluorescent intensity (MFI) of pBTK significantly increased in B cells and myeloid cells following BCR and FcγRII/III stimulation, respectively (Supplementary Fig. S3B). Importantly, BTK activation was reduced in FcRγ-deficient myeloid cells infiltrating orthotopic PDAC tumors (Fig. 3E). As macrophage depletion via administration of a depleting colony-stimulating factor (CSF1) antibody (αCSF1 mAb; refs. 9, 20, 23; Supplementary Fig. S3C) and B-cell depletion through loss of the *JH* locus (Fig. 2C) significantly reduced PDAC tumor growth *in vivo*, we postulated that BTK<sup>+</sup> B cells and macrophages were promoting PDAC growth.

### PI3Kγ Activates Macrophage BTK to Promote PDAC Growth

As PI3Ks can activate BTK to promote phospholipase C (PLC)γ-dependent signaling in hematopoietic cells (21, 24), we explored the roles of BTK and PI3Ks in B cell-macrophage interactions during PDAC tumorigenesis. Four unique isoforms of class I PI3Ks (p110α, β, δ, and γ) regulate PI3K signaling in cells (2). We found that primary murine macrophages express high levels of BTK and PI3Kγ (p110γ), but lower levels of PI3Kδ (p110δ), whereas B cells express high levels of BTK, PI3Kα (p110α), and PI3Kδ, but low levels of PI3Kγ; neoplastic PDAC tumor cells express only PI3Kα and PI3Kβ (p110β; Fig. 3F; Supplementary Fig. S3D). As PI3Kγ and BTK are similarly expressed in myeloid cells, and the PI3K isoform p110γ selectively promotes PLCγ-dependent integrin α4β1 activation leading to myeloid cell recruitment to tumors (25), we speculated that PI3Kγ might activate BTK to promote myeloid cell recruitment during PDAC progression.

To characterize the interactions between PI3Kγ and BTK in myeloid cells, we first compared their roles in mediating integrin α4β1 activation and cell adhesion, processes that are required for myeloid cell trafficking into tumors (25, 26). To achieve this, we used pharmacologic inhibitors and siRNA-mediated knockdown of *Btk*, as well as pharmacologic and genetic disruption of p110γ (PI3Kγ). To inhibit BTK, we utilized an FDA-approved BTK inhibitor, PCI-32765 (ibrutinib; ref. 27). PCI-32765 has an IC<sub>50</sub> of 0.5 nmol/L for BTK and exhibits cross-reactivity to BLK and BMX (IC<sub>50</sub> = 0.5 nmol/L), ITK (IC<sub>50</sub> = 11 nmol/L), and TEC (IC<sub>50</sub> = 78 nmol/L; ref. 22). To inhibit p110γ, we utilized the investigational PI3Kγ/δ inhibitor TG100-115, which has an IC<sub>50</sub> of 83 nmol/L for PI3Kγ, 238 nmol/L for PI3Kδ, and >1,000 nmol/L for PI3Kα and PI3Kβ (25, 28).

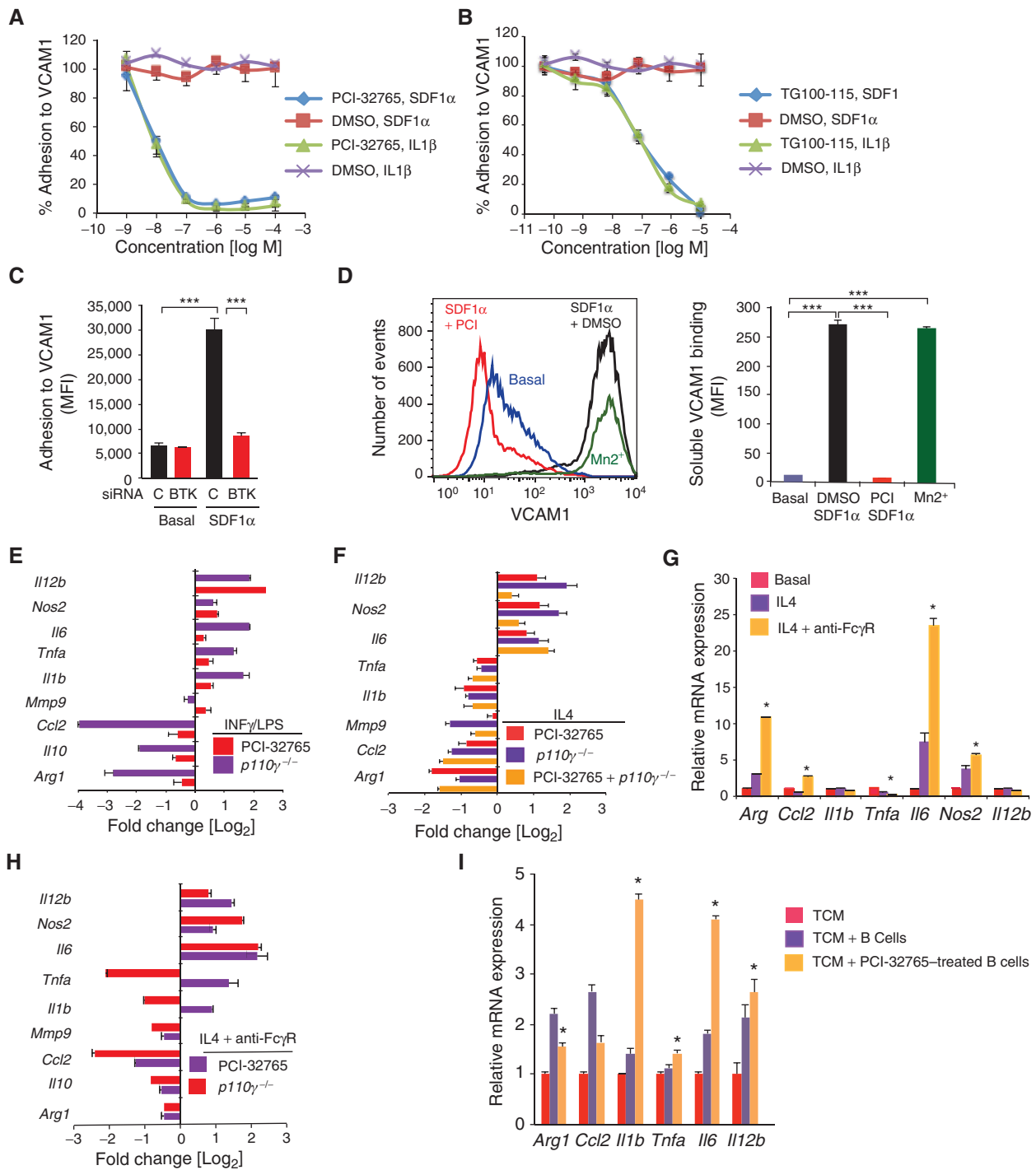
We previously found that stromal cell-derived factor 1 (SDF1)α and IL1β induce adhesion of primary macrophages to vascular cell adhesion protein (VCAM)1-coated surfaces (21–23); both PCI-32765 and TG100-115 significantly suppressed SDF1- and IL1β-mediated cell adhesion, with IC<sub>50</sub> values of 10 nmol/L and 100 nmol/L, respectively (Fig. 4A and B). In support of these findings, *Btk* knockdown (Supplementary Fig. S4A), similar to *p110γ* deletion and knockdown (21–23), suppressed adhesion of primary myeloid cells to VCAM1

(Fig. 4C). PCI-32765 and p110γ inhibition also suppressed CD11b<sup>+</sup> myeloid cell adhesion to vascular endothelial cell (EC) monolayers (Supplementary Fig. S4B), and PCI-32765 also suppressed B-cell adhesion to EC (Supplementary Fig. S4C). In addition, siRNA-mediated knockdown of PLCγ2, a key BTK and PI3Kγ signaling intermediate (21, 25; see schematic in Fig. 3A and B), suppressed myeloid cell adhesion (Supplementary Fig. S4D and S4E). Together, these findings support the hypothesis that PI3Kγ, BTK, and PLCγ2 regulate similar signaling pathways in myeloid cells that contribute to PDAC progression.

Based on these findings, we surmised that PI3Kγ might activate a BTK-signaling pathway to promote integrin α4β1 activation on macrophages prior to cell adhesion. In support of this, BTK inhibition, similar to PI3Kγ inhibition (25), suppressed integrin activation, as measured by cytokine-induced fluorescent VCAM1 binding to myeloid cells (Fig. 4D). In this assay, whereas VCAM1 binding to macrophages was rapidly stimulated by SDF1α or positive control Mn<sup>2+</sup>, VCAM1 binding was completely inhibited in the presence of PCI-32765 (Fig. 4D). Because BTK activation, as measured by autophosphorylation on Y223, was suppressed in macrophages lacking PI3Kγ (p110γ<sup>-/-</sup>; Supplementary Fig. S4F), we concluded that PI3Kγ activates a BTK-PLCγ2 pathway to promote integrin activation, myeloid cell adhesion and, potentially, myeloid cell recruitment to tumors *in vivo*.

Because we previously reported that PI3Kγ regulates protumorigenic properties of macrophages *in vivo* (25), we asked whether PI3Kγ activated BTK to promote protumor macrophage polarization. IFNγ/lipopolysaccharide (LPS) signaling induces macrophage expression of T<sub>H</sub>1 cytokines, including IL12, TNFα, IL6, IL1β, and nitric oxide synthase (NOS)2, and inhibits expression of T<sub>H</sub>2 immune-suppressive cytokines, including Arginase (ARG)1 and TGFβ *in vitro*, whereas IL4 signaling instead inhibits expression of T<sub>H</sub>1 cytokines and stimulates expression of T<sub>H</sub>2 factors *in vitro* (29, 30). We undertook an analysis of relative mRNA levels in IFNγ- or IL4-stimulated control and p110γ<sup>-/-</sup> or PCI-32765-treated macrophages by RT-PCR. Analysis of the change in mRNA levels between control and p110γ<sup>-/-</sup> or PCI-32765-treated macrophages indicated that genetic or pharmacologic inhibition of either PI3Kγ or BTK induced T<sub>H</sub>1 skewing of IFNγ/LPS-stimulated macrophages, as expression of *Il12*, *Il6*, *Tnfa*, *Il1b*, and *Nos2* was enhanced, and expression of *Arg1*, *Il10*, and *Ccl2* was reduced (Fig. 4E; Supplementary Fig. S4G and S4H). Inhibition of either PI3Kγ or BTK by pharmacologic or genetic approaches similarly induced T<sub>H</sub>1 skewing of IL4-stimulated macrophages while also inhibiting *Il1b* and *Tnfa* expression (Fig. 4F; Supplementary Fig. S4I). The combination of BTK inhibitors with PI3Kγ<sup>-/-</sup> macrophages had little additional effect on T<sub>H</sub>1 skewing of macrophage gene expression profiles (Fig. 4F), indicating that BTK and PI3Kγ regulate similar macrophage polarization pathways. Importantly, these observations also reveal that PI3Kγ and BTK similarly promote T<sub>H</sub>2 macrophage polarization and restrain T<sub>H</sub>1 polarization, indicating that these kinases may promote protumor T<sub>H</sub>2 macrophage polarization *in vivo*.

Because macrophage FcγR signaling promotes PDAC growth (Fig. 2), we investigated the consequences of FcγR cross-linking on macrophage polarization by incubating



**Figure 4.** FcR $\gamma$  signaling regulates BTK activation and mediates PDAC growth. **A** and **B**, effect of the BTK inhibitor PCI-32765 (**A**) and the p110 $\gamma$  inhibitor TG100-115 (**B**) versus vehicle control (DMSO) on IL1 $\beta$ - and SDF1 $\alpha$ -stimulated adhesion of primary myeloid cells to VCAM1 ( $n = 3$ ). **C**, effect of siRNA-mediated BTK knockdown on SDF1 $\alpha$ -stimulated myeloid cell adhesion to VCAM1 ( $n = 3$ ). **D**, effect of PCI-32765 (PCI) on SDF1 $\alpha$ -induced integrin  $\alpha 4\beta 1$  activation, as detected by binding of fluorescent VCAM1 to myeloid cells ( $n = 3$ ); left, FACS profiles and right, quantification of mean fluorescence intensity. Positive control, extracellular Mn<sup>2+</sup> treatment. **E** and **F**, Log<sub>2</sub> fold change of gene expression in (**E**) LPS/IFN $\gamma$  *in vitro*-polarized macrophages treated with PCI-32765 or p110 $\gamma$ <sup>-/-</sup> macrophages, (**F**) p110 $\gamma$ <sup>-/-</sup>, PCI-32765-treated, and p110 $\gamma$ <sup>-/-</sup> PCI-32765-treated IL4 *in vitro*-polarized primary murine macrophages ( $n = 3$ ). **G**, relative mRNA expression of macrophages polarized with IL4 or IL4 + FcR $\gamma$  cross-linking ( $n = 3$ ). **H**, Log<sub>2</sub> fold change of gene expression in IL4-stimulated FcR $\gamma$ -cross-linked p110 $\gamma$ <sup>-/-</sup> or PCI-32765-treated primary murine macrophages ( $n = 3$ ). **I**, relative mRNA expression of macrophages cocultured in p53 2.1.1 tumor cell-conditioned medium (TCM) with tumor-derived B cells ( $n = 3$ ) or PCI-32765-treated B cells. Data shown are mean  $\pm$  SEM of biological replicates and were validated in 3 or more separate experiments. Significance testing was performed by one-way ANOVA with Tukey *post hoc* testing for multiple pairwise testing or by the Student *t* test, where  $P > 0.05$  unless otherwise specified; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . For mRNA expression studies,  $P < 0.01$  unless otherwise indicated.

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macrophages with anti-Fc $\gamma$ R antibodies followed by secondary antibodies. Fc $\gamma$ R cross-linking of IL4-polarized macrophages enhanced T<sub>H2</sub> skewing, as macrophages displayed enhanced expression of key T<sub>H2</sub> factors, including *Arg1* and *Ccl2* (Fig. 4G). Importantly, inhibition of either PI3K $\gamma$  or BTK suppressed Fc $\gamma$ R-stimulated T<sub>H2</sub> cytokine expression and promoted T<sub>H1</sub> cytokine expression, with the exception that PI3K $\gamma$  inhibition suppressed, while BTK inhibition stimulated, *Tnfa* and *Il1b* expression (Fig. 4H). These minor differences in Fc $\gamma$ R-stimulated gene expression indicate that PI3K $\gamma$  regulates both BTK-dependent and BTK-independent gene expression pathways. Taken together, these results support the hypothesis that PI3K $\gamma$  activates BTK to promote macrophage T<sub>H2</sub> polarization.

We previously reported that B cell-derived Ig containing immune complexes stimulate Fc $\gamma$ R cross-linking to promote macrophage T<sub>H2</sub> cytokine expression (19). To determine if BTK regulated B cell-directed expression of T<sub>H2</sub> cytokines in macrophages, we cocultured PDAC-derived B cells with primary macrophages in coculture chambers *in vitro*. Under these conditions, B cells and macrophages were separated by a filter allowing the passage of only macromolecules between chambers. In the presence of PDAC cell-conditioned medium, tumor-derived B cells enhanced macrophage T<sub>H2</sub> skewing; however, pretreatment of PDAC-derived B cells with PCI-32765 suppressed this and instead enhanced expression of T<sub>H1</sub> cytokines (Fig. 4I). As B cell-derived immune complexes promote macrophage T<sub>H2</sub> cytokine expression (8), these results indicate that B cells promote the immunosuppressive, protumor properties of macrophages in a manner that depends on BTK in both B cells and macrophages.

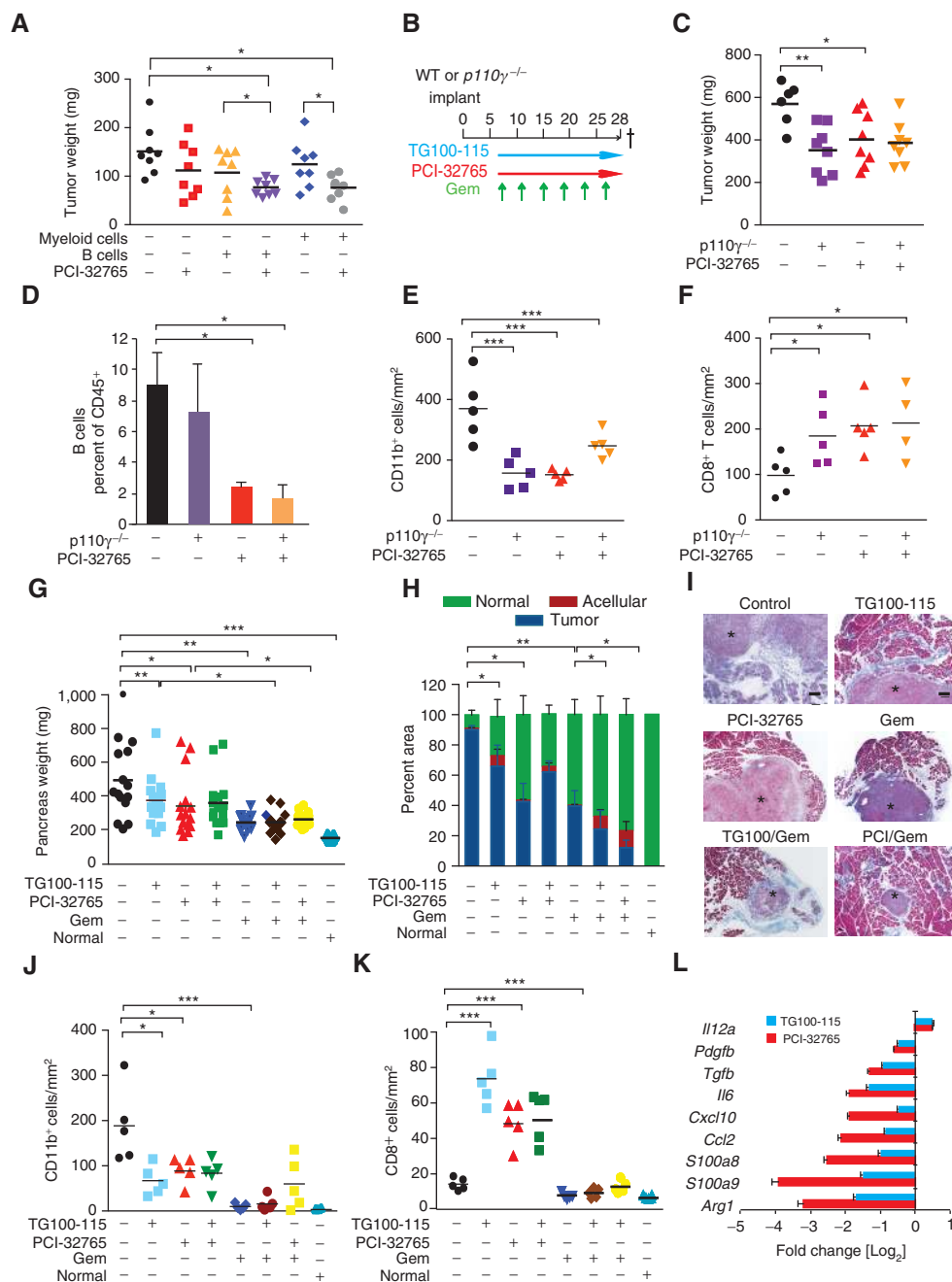
To evaluate the relative contributions of BTK in B cells and macrophages during tumor progression *in vivo*, B cells isolated from spleens of PDAC (p53 2.1.1)-bearing animals or Gr1<sup>+</sup>CD11b<sup>+</sup> myeloid cells (Supplementary Fig. S5A) isolated from tumors of PDAC (p53 2.1.1)-bearing animals were incubated with PCI-32765 or vehicle, mixed with tumor cells, and implanted in animals. Untreated B cells and myeloid cells had no significant effect on tumor growth, whereas pretreatment of B cells or myeloid cells with BTK inhibitor prior to implantation significantly suppressed tumor growth (Fig. 5A). These results support the hypothesis that BTK in both B cells and myeloid cells promotes PDAC tumor growth.

As neither BTK nor PI3K $\gamma$  inhibitors directly affected the viability of macrophages (Supplementary Fig. S5B) or PDAC tumor cells (Supplementary Fig. S5C and S5D), we evaluated whether PI3K $\gamma$  regulated BTK activity during PDAC growth *in vivo* by systemically treating WT and p110 $\gamma$ <sup>-/-</sup> mice bearing p53 2.1.1 PDAC tumors with or without PCI-32765 (Fig. 5B). Analogous to clinical scenarios observed in patients (31), mice receiving PCI-32765 exhibited transient lymphocytosis in the peripheral blood of tumor-bearing mice that resolved by the study termination (Supplementary Fig. S5E). PDAC tumor growth was similarly suppressed in p110 $\gamma$ <sup>-/-</sup> animals and in WT mice treated with PCI-32765, whereas PCI-32765 treatment of p110 $\gamma$ <sup>-/-</sup> mice had no additive effect on tumor growth (Fig. 5C). Importantly, p110 $\gamma$  deletion and PCI-32765 inhibitor treatment suppressed B-cell and CD11b<sup>+</sup> myeloid cell infiltration (Fig. 5D and E; Supplementary Fig. S5F) as predicted by results from the *in vitro* cell adhesion studies

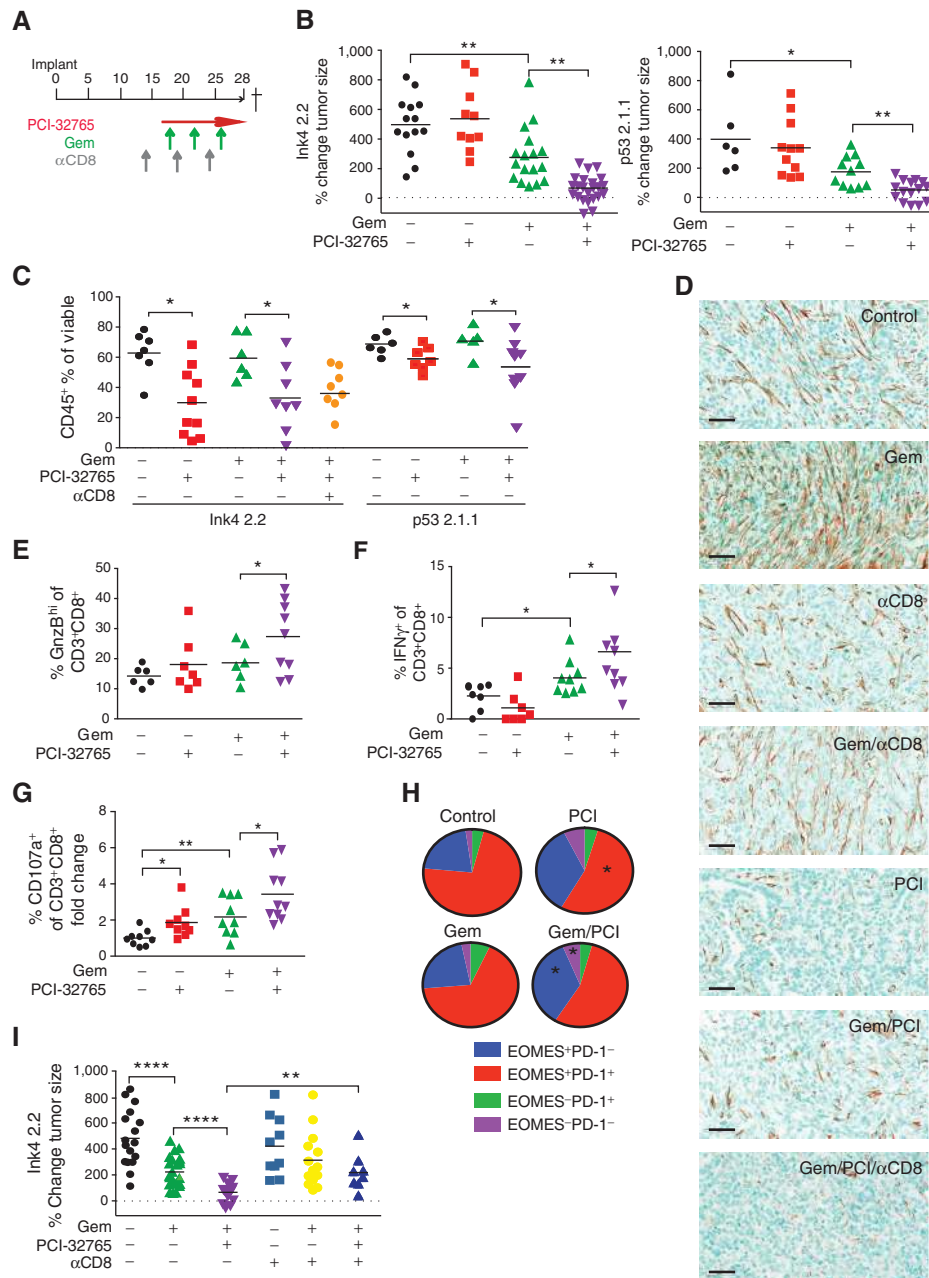
(Fig. 4A and B). p110 $\gamma$  deletion and PCI-32765 treatment also increased CD8<sup>+</sup> T-cell presence in tumors (Fig. 5F).

To evaluate the effect of combined treatment of PDAC tumors with PI3K $\gamma$  and BTK pharmacologic inhibitors, we treated mice bearing orthotopic p53 2.1.1 PDAC tumors with PI3K $\gamma$  and BTK inhibitors alone, together, or each in combination with gemcitabine (Gem; Fig. 5G). TG100-115 and PCI-32765 both suppressed PDAC tumor growth, as reflected in weights of pancreata 28 days after inoculation, but the combination of the two inhibitors had no additive effect (Fig. 5G). Gem monotherapy suppressed tumor growth as measured by pancreas weight, whereas Gem plus TG100-115 or Gem plus PCI-32765 had no additional impact on overall pancreas weight (Fig. 5G). However, histologic examination of end-stage tumors revealed little residual live tumor in pancreata treated with Gem plus PCI-32765 or TG100-115, indicating additive effects of the two therapies with Gem (Fig. 5H and I). Importantly, PI3K $\gamma$  and BTK inhibitors suppressed CD11b<sup>+</sup> myeloid cell infiltration (Fig. 5J; Supplementary Fig. S5G) and increased CD8<sup>+</sup> T-cell residency of tumors (Fig. 5J), similar to results observed in Fig. 5E and F. The combination of PI3K $\gamma$  and BTK inhibitors had no additional effects beyond those of each inhibitor alone, supporting the conclusion that PI3K $\gamma$  and BTK regulate the same pathway *in vivo* as well as *in vitro* (Fig. 4E-H). In addition, all Gem-treated pancreata exhibited fewer CD11b<sup>+</sup> and CD8<sup>+</sup> T cells than other treatments, as very little tumor tissue remained in these pancreata after 28 days (Fig. 5I-K). As PI3K $\gamma$  and BTK inhibition suppressed T<sub>H2</sub> and stimulated T<sub>H1</sub> cytokine expression in *in vitro* cultured macrophages (Fig. 4E-I), in the tumor microenvironment (Fig. 5L), and in tumor-derived myeloid cells (Supplementary Fig. S5H), these studies indicate that BTK and PI3K $\gamma$  regulate macrophage and T-cell programming *in vivo* as well as *in vitro*. Together these data support the conclusion that PI3K $\gamma$  and BTK promote a pathway leading to T<sub>H2</sub> polarization during PDAC progression. Furthermore, these data indicate that PI3K $\gamma$  and BTK inhibitors could be useful therapeutic approaches to treat early PDAC tumors.

Clinically, patients with PDAC typically present at late stage; thus, we sought to determine if therapeutic administration of PCI-32765 was efficacious in late-stage PDAC tumors as monotherapy, or in combination with Gem (Fig. 6A). PDAC growth in mice treated with combination PCI-32765/Gem administered at a late stage resulted in significantly reduced tumor size at end stage (Fig. 6B). Decreased tumor burden was associated with reduced presence of CD45<sup>+</sup> leukocytes (Fig. 6C; Supplementary Fig. S6A), associated with dynamic changes in the B-cell compartment whereby Gem treatment reduced the presence of memory B cells, BTK inhibitor (BTKi) resulted in increased frequency of T2 and B1b cells and reduced frequency of follicular and marginal zone B cells (Supplementary Fig. S2D), and decreased desmoplasia (Fig. 6D; Supplementary Fig. S6A). Importantly, we also observed a significant increase in the intratumoral frequency of granzyme B<sup>hi</sup>, IFN $\gamma$ <sup>+</sup>, and extracellular CD107a<sup>+</sup> CD8<sup>+</sup> T cells, indicative of their recent activation and degranulation (Fig. 6E-G; Supplementary Fig. S6A). In agreement with these data, there was an enhanced presence of programmed death (PD)-1<sup>+</sup>EOMES<sup>+</sup> late effector and PD-1<sup>-</sup>EOMES<sup>-</sup> short-term memory CD8<sup>+</sup> T-cell phenotypes (Fig. 6H; Supplementary Fig. S6B), that



**Figure 5.** PI3K $\gamma$  and BTK promote PDAC progression. **A**, tumor burden in mice implanted with admixed p53 2.1.1 cells together with PCI-32765- or vehicle-pretreated B cells or myeloid cells as compared with untreated or PCI-32765 after implantation treatment alone. **B**, schemas for early treatment schedule for PDAC-bearing mice with administration of PCI-32765 in drinking water (0.016% w/v) and/or the p110 $\gamma$  inhibitor TG100-115 (2.5 mg/kg b.i.d. given i.p.) beginning on day 7 after implantation, and gemcitabine (Gem; 15 mg/kg, i.v.) in p53 2.1.1-derived PDAC-bearing mice. Cross signifies end point or death. **C**, end-stage p53 2.1.1 PDAC tumor weights from syngeneic PI3K $\gamma$ -deficient ( $p110\gamma^{-/-}$ ) or WT mice treated with or without PCI-32765 ( $n = 10$ –15/ mice per experimental group). **D**, tumor-derived B cells as a percentage of CD45<sup>+</sup> cells in treated tumors from **C**, as determined by FACS analysis. **E**, number of CD11b<sup>+</sup> cells/mm<sup>2</sup> in treated tumors from **C**. **F**, number of CD8<sup>+</sup> T cells/mm<sup>2</sup> in treated tumors from **C**. **G**, effect of TG100-115, PCI-32765, gemcitabine (Gem), and combinations of these agents on growth to end stage of orthotopic p53 2.1.1 PDAC tumors ( $n = 10$ –15/ mice per experimental group) depicted as total pancreas weight after 3 weeks of treatment as compared with pancreas weight of tumor-naïve mice. **H**, percent residual tumor, normal acinar tissue, and acellular fibrotic tissue in pancreata of tumors depicted in **G**. **I**, representative photomicrographs showing Masson's trichrome stained images of PDAC tumors (\*) treated as shown reflecting quantitation in **G** and **H**. Scale bars, 200  $\mu$ m. **J**, number of CD11b<sup>+</sup> cells/mm<sup>2</sup> in treated tumors depicted in **C**, compared with normal pancreata. **K**, CD8<sup>+</sup> T cells/mm<sup>2</sup> in treated tumors depicted in **C**. **L**, log base 2 fold change in gene expression in tumors between control and TG100-115 or control and PCI-32765 treatments ( $n = 15$ ). mRNAs for which TG100-115-treated tumors showed statistically significant differences from control tumors are shown. Data shown are mean  $\pm$  SEM of biologic replicates and were validated in 3 or more separate experiments. Significance testing was performed by one-way ANOVA with Tukey *post hoc* testing for multiple pairwise testing or by Student *t* test, where  $P > 0.05$  unless otherwise specified, \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . ns, not statistically different. For **A**, **C**, **E**–**G**, **J** and **K**, each data point reflects an individual tumor with statistical means shown. For mRNA expression studies,  $P < 0.01$  unless otherwise indicated.



**Figure 6.** BTK-activated signaling regulates PDAC tumorigenesis. **A**, schemas for therapeutic administration of PCI-32765 (in drinking water [0.16% w/v]) beginning on day 14 after implantation and gemcitabine (Gem; 15 mg/kg, i.v., beginning on day 18) to late-stage PDAC tumor-bearing mice. 500  $\mu$ g depleting  $\alpha$ CD8 mAb antibody administered i.p. on days 15, 20, and 25. Cross signifies end point or death. **B**, percentage change in Ink4 2.2 (left) or p53 2.1.1 (right) tumor size from days 14 to 27 after implantation measured by ultrasonography and assessed following treatment with vehicle (-), PCI-32765, or Gem, and combinations as indicated. For Ink4 2.2, data from 3 independent experiments are shown, reflective of 5 independent experiments. For p53 2.1.1, data from one experiment are shown and are representative of 2 independent experiments. **C**, CD45<sup>+</sup> leukocyte frequency from end-stage tumors as a percentage of total viable cells determined by FACS analysis of single-cell suspensions of tumors from treatment groups indicated. Data from 2 independent experiments are shown and are representative of 5 independent studies. **D**, representative photomicrographs showing immunodetection of  $\alpha$ SMA in mice bearing end-stage Ink4 2.2 PDAC tumors treated as shown. Scale bars, 100  $\mu$ m. **E**, percentage of granzyme B-positive (GnzB) cells of CD3<sup>+</sup>CD8<sup>+</sup> cells as determined by FACS of end-stage Ink4 2.2 PDAC tumors treated as shown. Data from 2 independent experiments are shown and are representative of 4 independent studies. **F**, percentage of IFN $\gamma$ <sup>+</sup>CD8<sup>+</sup> T cells of CD3<sup>+</sup>CD8<sup>+</sup> cells as determined by intracellular FACS assessment of ex vivo-stimulated Ink4 2.2 PDAC tumors harvested from mice at day 23 after implant. Data from 2 independent experiments are shown. **G**, fold change of percent of CD107a<sup>+</sup>CD8<sup>+</sup> T cells of CD3<sup>+</sup>CD8<sup>+</sup> cells analyzed by FACS of Ink4 2.2 PDAC tumors harvested from mice at day 23 after implant. Data from 2 independent experiments are shown. **H**, FACS analysis of Ink4 2.2 tumors from day 23 time points for percentages of PD-1<sup>+</sup>EOMES<sup>+</sup> populations of CD3<sup>+</sup>CD8<sup>+</sup> T cells. Data from 2 independent experiments are shown. Statistical significance was determined using the Student t test or one-way ANOVA when analyzing more than two groups. **I**, percentage change in Ink4 2.2 tumor size from days 14 to 27 after implantation measured by ultrasonography and assessed following treatment with vehicle (-), PCI-32765, or Gem, and combinations in mice also administered depleting monoclonal antibodies for CD8<sup>+</sup> ( $\alpha$ CD8) T cells as indicated. Data from 2 independent experiments are shown. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . For **B-C** and **E-G** and **I**, each data point reflects an individual tumor, with statistical means shown.



correlated with  $T_H1$ -skewing of BTKi-treated tumors (Fig. 5L). This increased presence of effector  $CD8^+$  T-cell phenotypes was functionally significant as  $CD8^+$  T-cell depletion reinstated tumor growth to levels similar to Gem-treated mice (Fig. 6I; Supplementary Fig. S6C), without reinstating the  $CD45^+$  leukocyte infiltration (Fig. 6C) or desmoplasia (Fig. 6D) observed in untreated tumors. Taken together, these results indicate that myeloid cell PI3K $\gamma$  activates BTK, thereby promoting myeloid cell recruitment to PDACs and T cell-mediated immune suppression, and, importantly, indicate that pharmacologic inhibition of PI3K $\gamma$  or BTK activates a  $T_H1$  immune response that suppresses tumor growth.

## DISCUSSION

Pancreatic ductal adenocarcinoma is traditionally thought to be an immunologically silent malignancy, but recent therapeutic strategies have demonstrated that effective immune-mediated tumor cell death can be invoked to combat disease dissemination (32–34). The fundamental principle of these strategies is that  $CD8^+$  T cells can be mobilized to recognize and eliminate malignant cells if suppressive barriers to immunity are abated. Data presented herein reveal a critical role for B cells and Fc $\gamma$ -activated myeloid cells, and in particular macrophages, in regulating functionality of  $CD8^+$  T cells in PDAC, and identify BTK and PI3K $\gamma$  as key regulators of tumor immune suppression. Our studies demonstrate that both human and murine PDACs exhibit increased BTK activation in tumor-resident  $CD20^+$ ,  $CD11b^+$  and Fc $\gamma$ RI/III $^+$  cells relative to leukocytes in the periphery. BTK or PI3K $\gamma$  inhibition as monotherapy in early-stage PDAC, or in combination with Gem in late-stage PDAC, slowed progression of orthotopic tumors in a manner dependent on T cells. Coincident with slowed tumor growth, the percentage of  $CD8^+$  T cells with enhanced effector molecule expression increased in PDAC tumors that were functionally significant, as  $CD8^+$  T-cell depletion eliminated benefits of combined BTK inhibitor/Gem therapy. An increase in effector and memory  $CD8^+$  T-cell phenotypes was also observed midway through therapy, and thus consistent with other reports regarding  $CD8^+$  T-cell responses to various immunotherapies (35). Based on these data and reports from other groups (36, 37), we anticipate that targeted inhibition of BTK with therapies like ibrutinib may synergize with immune checkpoint inhibitors and/or select anticancer vaccines to further bolster T-cell activation for long-term durable antitumor immunity (36). Notably, this hypothesis is currently being tested in patients with PDAC and squamous cell carcinoma of the head and neck in two clinical trials (ClinicalTrials.gov Identifiers: NCT02436668 and NCT02454179).

Although protumorigenic activities of B cells have not been widely investigated, we and other groups have reported direct and indirect protumorigenic roles for tumor-infiltrating as well as peripheral B cells. In squamous carcinomas and melanomas, humoral immunity and IL10-secreting B1 cells induce  $T_H2$ -type programming of diverse myeloid cell types that foster tumor angiogenesis and  $CD8^+$  T-cell suppression (9, 19, 38, 39). In B cell-infiltrated prostate cancers (40), immunosuppressive B cells and plasmocytes promote survival of androgen-independent prostatic epithelial cells via

lymphotoxin-mediated NF $\kappa$ B regulation (41), and inhibit the efficacy of immunogenic CTX by IL10 and immune checkpoint-mediated suppression of CTLs (42). CTL suppression in these contexts is consistent with *in vitro* data revealing that IFN $\gamma$  release from  $CD8^+$  T cells and NK cells is increased when B cells are absent, whereas presence of B cells or B cell-derived IL10 is associated instead with reduced IFN $\gamma$  (43). Results reported herein lend further support for the role B cells play in mediating tumorigenesis and response to cytotoxic therapy and further identify BTK as a tractable target for anticancer therapy. Based on decreased presence of IgM $^lo$ CD23 $^+$ CD5 $^-$  follicular and IgM $^lo$ CD23 $^-$  memory B cells correlating with improved outcome and increased CTL bioactivity in BTKi-treated PDAC-bearing mice (Supplementary Fig. S2E), we anticipate operative activities for these B-cell subsets either in directly driving PDAC-associated T-cell suppression or in regulating CTL-mediated responses to cytotoxic therapy. At earlier stages of PDAC progression, however, IL35-producing B cells promote RAS-driven neoplasia (44) and B1b cell recruitment that are regulated by oxygen-sensing programs, that together slow early PDAC progression (45), and consistent with our results revealing dampened PDAC growth in B cell-deficient mice (Fig. 2). Together, these studies support both direct and indirect effects of B cells in regulating discrete aspects of tumorigenesis culminating in regulation of T-cell responses to neoplasia and cytotoxic therapy.

While identifying BTK as a B cell-intrinsic target, studies herein further identify macrophage BTK, and its regulation by Fc $\gamma$ - and PI3K $\gamma$ -mediated signaling, with subsequent effects on macrophage polarization, immune suppression, and induction of desmoplastic stroma. Although one prior study linked the class IA PI3Ks to BTK activation in B cells, recent studies have reported that these proteins regulate similar but distinct signaling pathways in B cells (33, 34). Our studies instead reveal the selective role of the class IB PI3K isoform PI3K $\gamma$  in regulating BTK to control the macrophage response to the tumor microenvironment. PDACs from BTKi and PI3K $\gamma$  inhibitor-treated mice exhibited a  $T_H1$ -polarized phenotype with mRNAs encoding stimulators of  $CD8^+$  T-cell cytotoxic activity. These observations are similar to antipathogen immunologic states and are necessary for immunogenic cell death of tumor cells (46), further supporting the hypothesis that BTK signaling regulates macrophage functionality and ultimately immunosuppression in PDAC tumors. Enhanced antitumor responses were observed when tumor-bearing mice were treated with BTKi therapy for an extended period of time commencing when PDAC tumors were small, as compared with later/larger stage tumors, supporting the general tenet that earlier detection and therapy in PDAC improves outcome.

Regarding BTK-regulated desmoplastic responses in PDACs, data presented herein are consistent with reported roles for macrophages in regulating muscle fibrosis and pancreatitis-associated fibrosis via pathways involving Arginine, TGF $\beta$ , MMP9, IL10, and other  $T_H2$ -type proteins (47, 48). Masso-Valles and colleagues also recently reported an important role for mast cells expressing BTK in regulating desmoplastic stroma of PDAC tumors (37). Although murine PDAC tumors evaluated herein did exhibit significant presence of mast cells, BTK inhibition did not alter the presence

of degranulating mast cells within tumor stroma (Supplementary Fig. S6D). Thus, if BTK regulates mast cell-induced desmoplasia, it likely does so via regulation of a protein secretion pathway, as opposed to a degranulation-mediated mechanism. Moreover, because CD8<sup>+</sup> T-cell depletion of PCI-32765/Gem-treated mice reversed the benefit of combination therapy (Fig. 6I), without affecting decreased desmoplasia of treated tumors (Fig. 6D), these data indicate that decreased PDAC tumor growth due to BTK inhibition is dependent on B-cell and/or myeloid cell reprogramming and bolstering of T<sub>H</sub>1-type immune responses, and independent of the desmoplasia that is characteristic of PDAC tumors.

Macrophage activation and polarization state are subject to a multitude of signals distinct in various tumor microenvironments. In mammary carcinomas, macrophages are recruited to tumor parenchyma by CSF1 expressed by mammary epithelial cells (49) and then polarized to a T<sub>H</sub>2 effector state following stimulation by IL4 produced by infiltrating CD4<sup>+</sup> T cells (50, 51). T<sub>H</sub>2-skewed macrophages in turn indirectly regulate CD8<sup>+</sup> T-cell functionality via their high-level expression of IL10, thereby impairing DC maturation and expression of IL12 (20). In contrast, macrophages in SCCs acquire a protumoral T<sub>H</sub>2-type phenotype following Ig-containing immune complex activation of FcγR receptors (19), which in turn represses expression of macrophage-derived chemokines required for CD8<sup>+</sup> T-cell infiltration (9). Whereas some of this same biology is preserved between pancreas and cutaneous microenvironments, other facets may play a more significant role in the pancreas, including mechanisms regulating B1b-cell recruitment into areas of hypoxia (45), and/or downstream of oncogenic RAS and CXCL13 (44). Future studies will determine why these tissue-specific differences exist, the appropriate patient populations likely to respond to these immunotherapy strategies, molecular mechanisms underlying paracrine BTK regulation of CD8<sup>+</sup> T-cell functionality, and the degree to which mast cells and macrophages differentially play a role downstream of BTK in regulating desmoplasia associated with PDAC tumorigenesis. As the BTK inhibitor ibrutinib is FDA approved for the treatment of leukemia (27), our studies indicate that BTK inhibitors could be rapidly evaluated as new therapeutic agents for pancreatic cancer.

## METHODS

### Freshly Isolated Human PDAC Samples and Peripheral Blood

All human samples for IHC, pathology, FACS, and plasma analysis were obtained and studied under informed consent in accordance with the Declaration of Helsinki and acquired through the Oregon Pancreatic Tumor Registry and IRB protocol #3609.

### Human PDA Tissue Microarray

Microarray data reflecting *CD20* and *Ig* mRNA in human tumor samples were queried from a commercially available data set (Bio-Express System, Gene Logic) originally generated on the Human Genome U133 Plus 2.0 Array (Affymetrix) and normalized by standard robust multichip average procedure. A single probe set of the highest variance among samples was chosen to represent *CD20* (228592\_at; MS4A1) and *Ig* (211430\_s\_at; IGHG1, IGHG2, IGHV4-31, IGHM), respectively. To ensure data consistency, results from

additional probe sets were compared with a single probe set. Median value was used to calculate fold change of expression in tumor tissue compared with normal tissue. Statistical analyses were performed using the Wilcoxon rank-sum test to compare mRNA expression levels to their corresponding normal tissue controls.

### Immunohistochemistry and Immunofluorescence

Detailed procedures are provided in the Supplementary Methods.

### Cell Lines

*Ink4 2.2* and *p53 2.1.1* cell lines were derived from primary PDAC tumors (FVB/N) of male transgenic *Pdx-Cre; LSL-Kras<sup>G12D</sup>* mice harboring null mutations in *Trp53* and *Trp16<sup>Ink4a</sup>* (10–16). Passage 3 of the cell lines was obtained in 2011 directly from the Hanahan laboratory, where they were derived and initially expanded. mCherry transfectants were expanded and frozen at low passage for use in the Coussens and Varner labs. Cells used in these studies were authenticated by gene expression in 2012 (RNA sequencing), whole exome analysis (in 2015), and BTK inhibitor sensitivity analyses (2013). All cell lines were tested for *Mycoplasma* contamination and grown in DMEM/10% FBS/1.0% penicillin and streptomycin on plastic coated with 50 μg/mL rat tail collagen I (BD Biosciences).

### Animal Husbandry and In Vivo Studies

All animal experiments were performed with approval from the Institutional Animal Care and Use Committees of the University of California, San Diego, or Oregon Health and Science University. Generation and characterization of B cell-deficient *JH<sup>-/-</sup>* (deletion in the J segment of the Ig heavy-chain locus and hence expressing no IgM or IgG and having no mature B cells), *FcRγ<sup>-/-</sup>*, and *p110γ<sup>-/-</sup>* mice have been described previously (17, 18, 25). All mice used for orthotopic implantation of PDAC cells were male FVB/n, 7 to 12 weeks of age. Mice receiving monoclonal antibodies for cellular depletion and/or cytokine neutralization were administered i.p. αCD8 (2.43, BioXcell) was administered on days 15, 20, and 25 after implantation at 500 μg/mouse; and αCSF1 (clone, BioXcell) was administered at 1.0 mg/mouse on day 21 and 500 μg/mouse on day 26. PCI-32765 (BTKi) was delivered *ad libitum* in drinking water at 0.16% w/v in a 2.0% β-hydroxycyclodextrin (Sigma) solution beginning on day 18, and continued until the end of the study. Gemcitabine was administered i.v. at 15 mg/kg on days 18, 22, and 26 after implantation. Alternatively, FVB/n mice inoculated orthotopically with p53 2.1.1 PDAC cells were treated from day 7 with PCI-32765 (0.016% w/v in 2.0% β-hydroxycyclodextrin *ad libitum* in drinking water) or TG100-115 (2.5 mg/kg i.p., b.i.d.) with or without gemcitabine (15 mg/kg on days 7, 10, 13, 16, 19, 22, and 25 after implantation). Prior to surgery, mice were anesthetized with isoflurane, and abdominal hair surrounding the surgical site were removed. A left abdominal flank incision (1.5 cm) was made, and spleen and adherent pancreas were exteriorized. PDAC cells (1.0 × 10<sup>3</sup> cells), mixed with 50% Matrigel and 50% serum-free DMEM in a total volume of 30 μL, were injected orthotopically into the tail of the pancreas of syngeneic 7-to-12-week-old male mice using a 30-gauge insulin needle, resulting in the appearance of a fluid bleb. Pancreas and spleen were then returned to their original position within the peritoneal cavity, followed by suture of the peritoneum and stapling of the skin. Staples were removed 7 days later, and tumor growth was monitored *in situ* using a Vevo 2100 small animal ultrasound (Visual Sonics) at days 14 and 27 following implantation. PDAC tumors were quantitated with ultrasound based on area (mm<sup>2</sup>) of the largest face of the tumor and percent change from days 14 to 27 was graphed, by weight in grams, or morphometrically using serial hematoxylin and eosin (H&E)-stained tissue sections by evaluating tumor area every 500 μm through the entire tissue depth and calculating the average

tumor area/section. Tissues were formalin-fixed or frozen in Tissue-Tek Optimal Cutting Temperature (O.C.T.) medium for histologic analysis. RNA was isolated from tissues flash-frozen in liquid nitrogen using the methods described for macrophage polarization.

### Peripheral Blood Analysis

Blood was collected via cardiac puncture at necropsy or by saphenous vein at specific time points during the study and placed into EDTA-coated tubes to prevent clotting. Complete blood count analysis was acquired on a Cell Dyn 3700 analyzer. Plasma was collected by spinning whole blood at 13,000 RPM for 5 minutes, then cell-free supernatant was collected and snap-frozen for later analysis.

### ELISA

Plasma from mice was thawed and diluted at 1:10,000 for assay of Ig levels and specific isotype. Concentrations of Ig in mouse plasma were determined using a standard curve and ELISA reagents from Southern BioTech. Human IgG was analyzed via the Human IgG Subclass Profile Kit from Novex-Life Technologies. Human samples were courtesy of the Oregon Pancreas Tumor Registry (OPTR). Data are shown as the fold increase relative to tumor-naïve mice.

### Statistical Considerations

For all *in vitro* studies, experiments were performed three or more times with 3 or more biological replicates per experiment group. *In vivo* experiments were performed at least twice, with 10 to 15 randomly assigned mice/group for orthotopic tumor studies in WT and p110 $\gamma$ <sup>-/-</sup> mice, and in mice treated with vehicle, PCI-32765, and TG100-115. A sample size of 15 mice/group provided 80% power to detect a mean difference of 1 standard deviation (SD) between two groups (based on a two-sample *t* test with two-sided 5% significance level). Prior to statistical analyses, data were examined for possible outliers using the Grubbs test. Data were normalized to the standard where applicable. Significance testing was performed by one-way ANOVA with Tukey *post hoc* testing for multiple pairwise testing or by parametric or nonparametric Student *t* test as appropriate. A two-sample *t* test (two groups) and ANOVA (multiple groups) were used when data were normally distributed, and a Wilcoxon rank-sum test (two groups) when data were not. All mouse studies were randomized for treatment by imaging all mice at day 14 after implantation and equally distributing mice into appropriate groups based on tumor size so that all groups began with a similar mean average (10–11 mm<sup>2</sup>) prior to treatment. Blinded assignment of mice into treatment groups, tumor measurement, and tumor analysis was performed by coding mice with randomly assigned mouse numbers, with the key unknown to operators until experiments were completed. Treatment studies were further blinded to evaluate tumor growth in response to therapy by having one investigator implant the mice and treat them while another investigator performed imaging at the indicated time points.

Additional detailed methods can be found in the Supplementary Methods.

### Disclosure of Potential Conflicts of Interest

M.A. Tempero reports receiving commercial research support from Pharmacyclics. J.A. Varner reports receiving a commercial research grant from Acerta LLC and is a consultant/advisory board member for the same. L.M. Coussens reports receiving a commercial research grant from Acerta Pharma and is a consultant/advisory board member for Pharmacyclics. No potential conflicts of interest were disclosed by the other authors.

### Authors' Contributions

**Conception and design:** A.J. Gunderson, M.M. Kaneda, N.I. Affara, M.A. Tempero, J.A. Varner, L.M. Coussens

**Development of methodology:** A.J. Gunderson, M.M. Kaneda, T. Tsujikawa, N.I. Affara, S. Gorjestani, B.Y. Chang, J.A. Varner, L.M. Coussens

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** A.J. Gunderson, M.M. Kaneda, A.V. Nguyen, N.I. Affara, B. Ruffell, S. Gorjestani, G. Kim, B. Sheppard, B. Irving, J.A. Varner, L.M. Coussens

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** A.J. Gunderson, M.M. Kaneda, T. Tsujikawa, A.V. Nguyen, N.I. Affara, B. Ruffell, S. Gorjestani, S.M. Liudahl, M.A. Tempero, J.A. Varner, L.M. Coussens

**Writing, review, and/or revision of the manuscript:** A.J. Gunderson, M.M. Kaneda, T. Tsujikawa, D. Hanahan, M.A. Tempero, B.Y. Chang, J.A. Varner, L.M. Coussens

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** A.J. Gunderson, T. Tsujikawa, M. Truitt, P. Olson, D. Hanahan, J.A. Varner, L.M. Coussens

**Study supervision:** A.J. Gunderson, J.A. Varner, L.M. Coussens

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