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Tumor-Stroma IL-1 β -IRAK4 Feedforward Circuitry Drives Tumor Fibrosis, Chemoresistance, And Poor Prognosis In Pancreatic Cancer

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Abstract

Targeting the desmoplastic stroma of pancreatic ductal adenocarcinoma (PDAC) holds promise to augment the effect of chemotherapy, but success in the clinic has thus far been limited. Preclinical mouse models suggest that near-depletion of cancer-associated fibroblasts (CAF) carries a risk of accelerating PDAC progression, underscoring the need to concurrently target key signaling mechanisms that drive the malignant attributes of both CAF and PDAC cells. We previously reported that inhibition of interleukin-1 receptor associated kinase 4 (IRAK4) suppresses NF- κ B activity and promotes response to chemotherapy in PDAC cells. In this study, we report that CAF in PDAC tumors robustly express activated IRAK4 and NF- κ B. IRAK4 expression in CAF promoted NF- κ B activity, drove tumor fibrosis, and supported PDAC cell proliferation, survival, and chemoresistance. Cytokine array analysis of CAF and microarray analysis of PDAC cells identified IL-1 β as a key cytokine that activated IRAK4 in CAF. Targeting IRAK4 or IL-1 β rendered PDAC tumors less fibrotic and more sensitive to gemcitabine. In clinical specimens of human PDAC, high stromal IL-1 β expression associated strongly with poor overall survival. Together, our studies establish a tumor-stroma IL-1 β -IRAK4 feedforward signal that can be therapeutically disrupted to increase chemotherapeutic efficacy in PDAC.

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INTRODUCTION

The heavily fibrotic tumor microenvironment (TME), also termed desmoplasia, is a salient feature of pancreatic ductal adenocarcinoma (PDAC) (1). Major components of the TME include a dense fibrotic matrix created by activated cancer-associated fibroblasts (CAFs), various subtypes of immune cells, vascular cells and nerve cells(2–4). The fibrotic matrix consists mostly of collagen, fibronectin and hyaluronan and other matricellular proteins that forms a high-pressure physical barrier that is believed to limit the delivery of chemotherapeutic agents(5–8). The desmoplastic TME and CAFs can promote PDAC cell proliferation, invasion, metastasis, treatment resistance and metabolic reprogramming (5, 9–12). Therefore, stromal-depleting strategies hold promise to improve the efficacy of chemotherapy, as shown in a phase 1 clinical trial where addition of a hyaluronan-degrading enzyme improved response to gemcitabine in patients with hyaluronan-high PDAC (13). On the other hand, targeting the SHH or JAK2-STAT pathways, both shown to be essential for PDAC desmoplasia in preclinical studies, unexpectedly failed to improve patient survival in clinical trials(14, 15). Moreover, preclinical mouse models demonstrated that near-complete stromal depletion could revert PDAC cells to a more primitive and aggressive state(16, 17). Therefore, therapeutic strategies that can effectively target both PDAC cells and stromal CAFs should continue to be explored.

Targeting the NF- κ B pathway could be a promising strategy. We recently reported that constitutive activation of Interleukin-1 Receptor Associated Kinase 4 (IRAK4), a master kinase that relays Toll-like/IL-1 receptor signaling, promotes chemoresistance of PDACs cells in an NF- κ B-dependent manner(18). However, the role of IRAK4 or NF- κ B in PDAC CAFs has not been reported. In a murine model of squamous cell carcinoma, cancer cells can activate NF- κ B and transform normal fibroblasts into tumor-promoting CAFs that secrete inflammatory cytokines including CXCL1, CXCL2, IL-1 β and IL-6(19). In the same report, CAFs isolated from PDAC tumors also shared similar secretory signature(19), suggesting NF- κ B activation may also be a contributing factor in the malignant attributes of CAFs. However, depletion of *IKK β* gene, whose product activates NF- κ B, using different CAF-specific promoters, had completely opposite effects on colon cancer progression in a colitis mouse model(20, 21). These conflicting findings highlight the need to study how NF- κ B affects CAF behaviors in a cancer-specific manner.

In this study, we made initial observation that PDAC CAFs showed robust phosphorylation of IRAK4 and NF- κ B, prompting us to hypothesize that IRAK4 may be a driver of NF- κ B in CAFs. However, systemic inhibition of MyD88, the binding adaptor that activates IRAK4, paradoxically induces stromal expansion and tumor progression through recruitment of dendritic cells in a genetically-engineered mouse model (GEMM) of PDAC(22). In this study, we sought to understand whether IRAK4 activates NF- κ B in PDAC CAFs, the role of IRAK4 in malignant attributes of CAFs specifically in abetting PDAC growth and chemoresistance, and molecular mechanism that activates CAF IRAK4. This information must be clarified before testing IRAK4 inhibitors in the clinic.

MATERIAL AND METHODS

(Please also see Supplemental Data)

Cell lines

All human cell lines were purchased from ATCC, which performs its own authentication by short tandem repeat DNA profiling. Human CAF-1, CAF-2 and CAF-3 were isolated from surgical specimens of patients who underwent Whipple procedure, and expanded using published method(23). SC00A5 and CAF-8 were purchased from Vitro Biopharma, which performs its own authentication. All CAFs were confirmed to express α -SMA by western blots, as well as by immunofluorescence (IF) to express α -SMA but not pan-cytokeratin. KPC2 cells were a kind gift from Dr. David DeNardo (Washington University in St. Louis, WUSTL), were published and authenticated by whole-exome sequencing(24). All cells were cultured in DMEM/10% FBS/1% Pen-Strep at 5% CO₂, 37°C incubators. Mycoplasma testing was performed every 6 months using MycoSEQ Detection kit (Applied Biosystems). All lines were used for fewer than 6 months after receipt or resuscitation from cryopreservation.

Human, murine PDAC samples and Tissue Microarrays (TMA)

FFPE PDAC samples were retrieved from the Department of Pathology and Immunology at WUSTL under IRB approval (#201404143). WUSTL PDAC TMA with survival data was constructed by Dr. Dengfeng Cao at WUSTL, was IRB approved and published (25). Patient consent was waived for both set of samples under IRB approval. Commercial PDAC TMA was from US BioMax (PA 2081). All studies were performed per ethical principles of Declaration of Helsinki. Stromal IL-1 β IHC intensities were scored independently by KHL, LL and DZ, and survival analysis was performed by JL in blinded manner.

Immunohistochemistry (IHC) and Immunofluorescence (IF)

IHC and IF staining were performed using the following antibodies: p-IRAK4 (T345S) (ABNOVA, A8A81:200), p-NF- κ B/p65 (S536) (Abcam ab86299, 1:200), α -SMA, Ki-67, cleaved caspase-3 antibodies (Cell Signaling), IL-1 β (Proteintech, 16806-1-AP, 1:100). Sirius Red staining was performed and quantified as described(24). Unless otherwise specified, all quantification was performed under fluorescence or light microscopy on ten 20 $\times$ power fields/tumor, and data were presented as mean $\pm$ SEM.

Plasmids and creation of stable cell lines

Stable knockdowns were generated using pSuperRetro as described(18). Target sequences: shIRAK4#1: TTCAGTAGTAATGTCAACC ; shIRAK4#2: GCCTAATGGTTCATTGCTA; shIL-1R#1: GCCAAGAATACACATGGTATA, shIL-1R#2: GTGCTTAATATATCGGAAATT.

Drugs and reagents

Gemcitabine was purchased from the Siteman Cancer Center Pharmacy. Details of inhibitors were: IRAK1/4 inhibitor (Sigma, cat# I5409), AS2444697 (Tocris, cat#5430), IMD-0354

(Tocris, cat#2611), anti-mouse IL-1 β neutralizing antibody (Invivogen, clone 7E3), anti-human IL-1 β neutralizing antibody (Invivogen, clone 4H5).

Microarray Analysis

See Supplemental Data.

IL-1 β ELISA

Quantification of IL-1 β in conditioned media was performed using Human IL-1 beta/IL-1F2 Quantikine ELISA Kit from R&D Systems per manufacturer's protocol.

NF- κ B and Caspase 3/7 reporter assays

These were done using Dual-Glo® Luciferase Assay System, and Caspase-Glo® 3/7 Assay (Promega), and read with Synergy H4 Hybrid Microplate Reader.

Xenograft tumorigenesis assay

All animal experiments were conducted under approval by WUSTL IACUC protocol (#20130191). *IRAK4*-null mice were purchased from EUCOMM and genotyped as recommended(26). All co-injection experiments were performed using PDAC cells: CAFs at 1:2 ratio.

Statistical Analyses

All results, when applicable, were expressed as the means $\pm$ SEM. Statistical analysis was performed using the Prism 6 software program. Unpaired student's two-tailed t-tests were used to compare two groups when appropriate. For multiple groups, one-way ANOVA analysis with Tukey's post-test were used. P values <0.05 were considered as statistically significant. Cox proportional hazards models were used to evaluate the relationships between clinical characteristics and overall survival. Kaplan-Meier curve was generated using SAS version 9.4 (SAS Institute, Cary, NC) and analyzed by log rank tests.

RESULTS

IRAK4 is constitutively phosphorylated and drives NF- κ B activity in PDAC CAFs

First, we observed robust p-IRAK4 (T385) staining in α -SMA⁺ spindle-shaped fibroblasts within the stroma of human and murine PDAC tumors, and Capan-1 xenograft tumors, using both immunohistochemistry (IHC) and co-immunofluorescence (IF) (Fig. 1A–1D, Suppl. Fig. 1A). Notably, p-IRAK4 staining is present in the stroma of four out of ten pancreatitis samples. For further confirmation, we investigated the p-IRAK4 status of four early-passaged (<10 passages) CAF lines isolated from human surgical PDAC tumors by western blots, and found all four CAF lines to have significantly higher p-IRAK4 levels compared to normal human foreskin (hFB) or pancreatic fibroblasts (SC00A5). These PDAC CAFs also had higher p-p65 (or RelA) levels compared to normal fibroblasts, and showed strong α -SMA expression (Fig. 1E, Suppl. Fig. 1B). To determine whether IRAK4 activates NF- κ B in CAFs, we treated these CAF lines with a dual IRAK1/4 inhibitor, or an IRAK4-specific inhibitor, AS2444697(27). AS2444697 is 30 times more specific in inhibiting IRAK4 than

IRAK1, and was shown to prevent kidney inflammation and production of various proinflammatory cytokines in nephrectomized rats (27). As confirmation of specificity, AS2444697 suppressed anchorage-independent (AI) growth of Capan-1 and PANC-1 cells, but had little effect on IRAK4-silenced counterparts (Suppl. Fig 1C). Both inhibitors dose-dependently suppressed p-IRAK4 and p-p65 levels in CAF-2 and CAF-8, but not in SC00A5 fibroblasts (Fig. 1F). However, α -SMA protein levels were unaffected by IRAK4 inhibitors, indicating IRAK4 inhibition alone is unable to radically alter the identity of CAFs. Overexpression of wild-type IRAK4 increased p-p65 levels in hFB and SC00A5, whereas kinase-dead IRAK4 mildly elevated p-p65 in SC00A5, perhaps through engaging endogenous IRAK4 proteins, but not in hFB (Fig. 1G). Silencing IRAK4 by two different shRNAs suppressed p-p65 levels in CAF-2 and CAF-8 (Fig. 1H). Overall, these findings showed that IRAK4 is activated and drives NF- κ B activity in PDAC CAFs.

CAF IRAK4 supports PDAC cell proliferation, migration and tumorigenesis

CAFs promote tumor progression partly by supporting proliferation of PDAC cells(9). To understand the role of CAF IRAK4 in this aspect, we co-cultured MIA Paca-2 cells stably co-expressing RFP and firefly luciferase with CAF-2 or CAF-8 expressing GFP and either a scramble control shRNA or two different shIRAK4s. We found that silencing of IRAK4 significantly impaired the ability of CAFs to promote MIA Paca-2 proliferation, as assayed by luciferase titer and abundance of RFP⁺ cells (Fig. 2A, Suppl. Fig. 2A). However, neither shIRAK4 nor IRAK4 inhibitor affected proliferation of CAFs in 2D culture condition (Suppl. Fig 2B, 2C). We also found that conditioned medium (CM) from shIRAK4 CAF-2/8 was defective in promoting wound closure and transwell migration of MIA Paca-2 cells, compared to CM from control CAF-2/8 (Fig. 2B, 2C). Consistent with these findings, shIRAK4 CAF-2/8 were severely impaired in supporting tumorigenesis when co-injected subcutaneously with MIA Paca-2 cells into nude mice (Fig. 2D). Immunofluorescence analysis of resected MIA Paca-2 tumors showed that the ability of CAF-2/8 to promote MIA Paca-2 cell proliferation *in vivo*, as discerned by abundance of dual Ki-67⁺/pan-cytokeratin⁺ cells in tumor sections, was abrogated when IRAK4 was silenced (Fig. 2E). Together, these findings suggest an essential role for CAF IRAK4 in supporting PDAC cell proliferation *in vitro* and *in vivo*.

CAF IRAK4 contributes to tumor fibrosis

Next, we studied the role of CAF IRAK4 in promoting tumor fibrosis, which has not been previously reported. We found that AS2444697 significantly impaired the collagen-contracting ability of CAF-2 and CAF-8 *in vitro* (Fig. 3A). Compared to SC00A5, both CAF lines expressed higher level of collagen 1 mRNA, which was lowered by shIRAK4s (Fig. 3B). Accordingly, IRAK4 knockdown abrogated the fibrogenic ability of CAF-2 and CAF-8 when co-injected with MIA Paca-2 cells into nude mice, as discerned by Sirius Red⁺ area in tumor sections (Fig. 3C). However, this co-injection model does not account for the contribution of mouse fibroblasts within the tumor bulk. To overcome this caveat, we injected KPC2, a murine PDAC line derived from the *p48-Cre/p53^{fllox/WT}/LSL-KRas^{G12D}* model model(24), into syngeneic wild-type or *IRAK4*-null C57BL/6J mice. We again observed significantly lower final weights of KPC2 tumors grown in *IRAK4*-null mice, compared to those grown in wild-type mice (Fig. 3D, Suppl. Fig. 3). Analyses of KPC2

tumor sections showed significantly reduced number of proliferating KPC2 cells (discerned by dual pan-cytokeratin⁺ and Ki-67⁺ staining) and degree of fibrosis (by Sirius Red staining) of tumors injected into *IRAK4*-null mice (Fig. 3E, 3F). However, tumor weights, KPC2 cell proliferation and tumor fibrosis could be rescued by co-injection with wild-type, but to a much smaller degree *IRAK4*-null, murine embryonic fibroblasts (MEFs) (Fig. 3D, 3E and 3F). These findings substantiate our previous report that systemic treatment with IRAK1/4 inhibitor significantly reduce α -SMA content of PDAC tumors(18), and provided a direct evidence for CAF IRAK4 in supporting PDAC fibrosis.

Autocrine IL-1 β activates IRAK4 in CAFs

A key mechanism by which CAFs induce tumor fibrosis is via secretion of inflammatory cytokines(28). To understand the role CAF IRAK4 in this aspect, we performed cytokine array analysis on CM collected from control or IRAK4-silenced CAF-2 cells. Using densitometry, we found that several cytokines/chemokines were downregulated by at least 50% in the CM from shIRAK4 CAFs, including IL-1 β , CXCL1/2/3, CXCL5, OPG, IL-6, IL-8, IL-12 and CCL2 (Fig. 4A). We validated these findings by qPCR of CAF-2 and CAF-8 cells infected with two different shIRAK4s or treated with two different IRAK4 inhibitors (Fig. 4B, Suppl. Fig 4). Interestingly, similar findings were seen with SC00A5 (Suppl. Fig 4), indicating a conserved role of IRAK4 in cytokine production in fibroblasts. Of these secreted factors, IL-1 β caught our attention because: (a) its transcription was the most significantly impaired following genetic or pharmacologic IRAK4 suppression; (b) as a known activator of IRAK4 it may activate CAF IRAK4 in an autocrine manner(29) and; (c) while the major source of IL-1 β in PDAC tumors has been attributed to PDAC cells(30–32), tumor-infiltrative myeloid cells(33), or adipocytes in an obesity-induced PDAC mouse model(34), contribution of IL-1 β from CAFs remains understudied. To this end, we confirmed presence of robust IL-1 β staining in α -SMA⁺ CAFs by IHC in 96 out of 117 PDAC samples in a commercial PDAC TMA (Fig. 4C). Consistently, we observed co-staining of IL-1 β and α -SMA by IF in stromal cells of independent human surgical and murine PDAC samples (Fig. 4D). Consistent with our qPCR data (Fig. 4B), treatment with AS2444697 or shIRAK4 significantly reduced IL-1 β concentration measure by ELISA in CAF-2 and CAF-8 CM (Fig. 4E). Furthermore, treatment of CAF-2 with an neutralizing anti-human IL-1 β antibody or an IL-1R antagonist, Anakinra, led to significant reduction of p-IRAK4 and p-p65 levels by western blots (Fig. 4F). These results suggest that PDAC CAFs maintain an autocrine IL-1 β -IRAK4 loop to sustain NF- κ B activity.

PDAC cells secrete IL-1 β to activate CAF IRAK4

Secreted factors from neighboring PDAC cells are another source of ligands that can activate IRAK4 in CAFs. Supporting this notion, incubation of normal pancreatic fibroblasts SC00A5 with CM from PDAC lines upregulated p-IRAK4 and p-p65 levels to various extents that appear to correlate with the abundance of IL-1 β in these CM (Fig. 5A, 5B). Notably, CM from MIA Paca-2 further promoted production of IL-1 β mRNA from CAF-2 and CAF-8 cells (Fig. 5C). These findings suggest that PDAC CM contains factors that can trigger the IL-1 β -IRAK4 autocrine loop in CAFs. Because we previously reported that IRAK4 controls secreted factors from PDAC that stimulate CAF invasion and migration(18), we decided to first investigate genes whose expressions are significantly affected by IRAK4

activity and whose products are known to activate IL-1R and the TLRs. To this end, we compared microarray analyses of 34,595 genes in Capan-1 and PANC-1 cells treated with DMSO or IRAK1/4 inhibitor. Again, we found IL-1 β to be downregulated by at least 1.5 fold in both cell lines (Fig. 5D). None of the other known activators of IRAK4 including IL-18, heat shock proteins or HMGB1 showed significant changes in an IRAK1/4-dependent manner. These results imply IL-1 β as the main factor in PDAC CM that activates CAF IRAK4. Indeed, addition of anti-human IL-1 β neutralizing antibody effectively blocked the ability of MIA Paca-2 or Capan-1 CM to phosphorylate IRAK4 and p65 in SC00A5 cells (Fig. 5E), and significantly impaired the ability of MIA Paca-2 CM to promote gel-contraction by SC00A5 cells (Fig. 5F). Consistent with these findings, SC00A5 cells stably expressing two separate shRNAs against IL-1R were unable to upregulate p-IRAK4 and p-p65 when treated with MIA Paca-2 CM (Fig. 5G). These findings suggest that IL-1 β secreted from PDAC cells can activate CAFs through the IL-1R-IRAK4 axis to promote their fibrogenic function.

CAF IRAK4 drives IL-1 β secretion to promote PDAC cell NF- κ B activity and survival

While PDAC cells can promote CAF function through paracrine factors, CAFs are known to reciprocally promote survival and chemoresistance of PDAC cells through secreted factors and exosomes (9, 35). However, the role of CAF IRAK4 in supporting PDAC cell survival is unknown. To this end, we found that CAF-2 and CAF-8 CM significantly promoted the survival of PDAC cells cultured in the absence of serum, a condition that mimic the nutrient-poor TME of PDAC (Fig. 6A). However, the pro-survival effect was lost in CM collected from IRAK4-silenced CAFs. Mechanistically, we found that CM from control, but not shIRAK4 CAF-2 cells significantly upregulated NF- κ B activity of MIA Paca-2 cells, indicating that CAF IRAK4 controls secreted factors that promotes PDAC cell survival (Fig. 6B). Furthermore, because activated NF- κ B also confers chemoresistance of PDAC cells (18, 36), we next asked whether CAF IRAK4 controls secreted factor to modulate resistance of PDAC cells to gemcitabine, a key chemotherapeutic agent used for PDAC treatment. Indeed, CM from control CAF-2, but not SC00A5 or shIRAK4 CAF-2, was able to protect MIA Paca-2 and CFPAC-1 cells from killing by gemcitabine *in vitro*, as evidence by significantly enhanced IC₅₀ values of gemcitabine (Fig. 6C). Mechanistically, CM from control CAF-2 and CAF-8, but not their shIRAK4 counterparts or SC00A5, was able to rescue MIA Paca-2 cells from gemcitabine-induced apoptosis, as measured by caspase 3/7 reporter activity (Fig. 6D) and double Annexin V and PI staining by flow cytometry (Suppl. Fig. 5). We previously showed that IRAK4 drives NF- κ B and chemoresistance in PDAC cells (18), and since IRAK4 is activated by IL-1R (37), we hypothesize that IL-1 β is the key factor in CAF CM that drives PDAC cell survival. To test this possibility, we added neutralizing anti-human IL-1 β antibody to CAF-2 CM, and found that anti-IL-1 β antibody dose-dependently blocked the ability of CAF-2 CM to promote survival of PDAC cells in serum-free condition (Fig. 6E), and stimulate NF- κ B in MIA Paca-2 cells (Fig. 6F). Together, these results suggest that CAF IRAK4 support PDAC cell survival and chemoresistance at least partly through secreted IL-1 β .

Disruption of tumor-stroma IL-1 β -IRAK4 circuitry attenuates tumor fibrosis and augments the effect of gemcitabine *in vivo*

Tumor fibrosis and enhanced NF- κ B activity are two main factors that curb the effectiveness of chemotherapy in PDAC. Our data so far led us to hypothesize that PDAC cells acquire these feats through forming an IL-1 β -IRAK4 feedforward loop with CAFs. Supporting this hypothesis, we found that Capan-1 cells co-injected with CAF-2 formed significantly larger tumors (Fig. 7A), which were characterized by significantly higher number of proliferating Capan-1 cells (Fig. 7B, Suppl. Fig. 6A) and infiltrative α -SMA⁺ fibroblasts (Fig. 7C, Suppl. Fig. 6B). The pro-tumorigenic effect of CAF-2 were abolished when either IRAK4 or IL-1R was silenced by shRNAs (Fig. 7A–C). These findings provided a rationale for combining a neutralizing anti-IL-1 β (nIL-1 β) antibody with chemotherapy, which to our best knowledge has not been investigated. To test this regimen, we used KPC2 cell model that forms fibrotic tumors when injected subcutaneously in syngeneic C57BL/6J mice(24). We chose to use a pure murine model over human xenograft models because neutralizing anti-IL-1 β antibodies are species-specific and anti-mouse IL-1 β antibody should effectively block IL-1R signaling in both PDAC cells and CAFs. Consistent with our hypothesis, combined administration of anti-mouse nIL-1 β antibody and gemcitabine, but neither alone, significantly reduced KPC2 tumor weight (Fig. 7D). Notably, AS2444697 alone or in combination with gemcitabine were effective in curbing KPC2 tumor growth, providing further support for targeting the IL-1R-IRAK4 axis in PDAC. Histologic analyses showed that tumors treated with both nIL-1 β and gemcitabine had significantly less proliferating pan-cytokeratin⁺/Ki-67⁺ cells (Fig. 7E, Suppl. Fig 6C), more cleaved-caspase 3⁺ apoptotic cells (Fig. 7F, Suppl. Fig. 6D), and less fibrosis (Fig. 7G Suppl. Fig. 6E). Notably, AS2444697 alone or in combination with gemcitabine were effective in suppressing tumor cell proliferation, fibrosis, and augmenting apoptosis in this model. Together, our data suggests IL-1 β as a driver of PDAC chemoresistance and fibrosis through activating IRAK4 and NF- κ B in CAFs. Consistently, high stromal IL-1 β IHC staining in surgical PDAC samples is associated with significantly inferior overall survival in a cohort of 125 patients who underwent curative resection at our institution (Fig. 7H). Multivariate analysis showed high stromal IL-1 β to be an independent poor prognostic factor after surgical resection (Suppl. Table 1). On this basis, we proposed a model in which PDAC cells and CAFs conspire to generate a feedforward IL-1 β -IRAK4 circuitry that propel tumor fibrosis, chemoresistance, and poor prognosis (Fig. 7I).

DISCUSSION

The prognosis of patients with advanced PDAC remains extremely poor due to the lack of effective treatment. Current standard treatment consists of strong chemotherapy which trades a few months of survival extension for significant toxicities (38). The therapeutic breakthrough brought forth by immune checkpoint blockade has not materialized yet in PDAC patients. Overcoming the intrinsic resistance mechanisms and the extrinsic protective TME thus represent the most logical strategies to improve the effect of chemotherapy and patient outcome (39–42).

We previously reported that targeting IRAK4 sensitizes PDAC cells to chemotherapy by attenuating NF- κ B activity (18). In this study, we investigated the mechanism and role of IRAK4 activation in CAFs, with the goal of understanding whether targeting IRAK4 in this cell population, which is shown to confer chemoresistance, is a promising therapeutic strategy. We showed that IRAK4 drives NF- κ B activity and secretion of several inflammatory cytokines from CAFs. Contrary to the detrimental effect of MyD88 inhibitor as shown from the Miller lab (22), our studies showed that targeting stromal IRAK4 reduces tumor fibrosis, PDAC cell proliferation, survival and augments the effect of gemcitabine. This discrepancy could be explained by differences in experimental models (xenograft model versus GEMM), or different outcome from inhibiting MyD88 versus IRAK4. In subsequent reports from the Miller lab, ligation of TLR9 or TLR7, which both signal through IRAK4, greatly accelerates chemical-induced PDAC progression in GEMM through stromal expansion(43, 44). These findings establish an important role of the TLR signaling pathway in PDAC progression, but at the same time underscore the need to clearly delineate the role and consequence of targeting different signaling components of this pathway in a cell-type dependent manner, which our current study has helped achieved. Nonetheless, clearer delineation of the role of stromal IRAK4 in PDAC development should be pursued through systemic IRAK4 inhibition in PDAC GEMM, and generation of conditional *IRAK4*-null mice.

Mechanisms that reprogram normal fibroblasts into CAFs remain largely unclear, but recent evidence showed tumor-induced DNA methylation to be involved(45). Our data showed that IRAK4 inhibition does not affect α -SMA level or impair proliferation of CAFs *in vitro*, but significantly impairs their fibrogenic and pro-tumorigenic functions *in vivo*. These results indicate sustained IL-1R-IRAK4 activation is one of many mechanisms that support the malignant feats of CAFs, and other tumor-stroma interacting mechanisms are involved to fully convert normal fibroblasts into a pro-tumorigenic state. Corroborating this notion, we provided evidence, based on our single institution PDAC patients, that high stromal IL-1 β expression portends poor overall prognosis.

Our study identified IL-1 β as a cytokine that activates IRAK4 in CAFs. We arrived at this conclusion via investigating secreted factors whose expression is IRAK4-dependent and can reciprocally activate IRAK4. Previous reports from the Schaffer lab showed that tumor-stroma IL-1 β circuitry drives resistance of PDAC cells to etoposide *in vitro* through NF- κ B activation (31, 32). Our current study further supports these findings and provides additional mechanistic insights by (1) linking IL-1 β signaling to IRAK4, thereby establishing IRAK4 as another therapeutic target; (2) demonstrating that blocking IL-1 β renders PDAC cells sensitive to gemcitabine *in vivo*, the chemotherapy used in the clinic for PDAC patients, (3) providing *in vitro* and *in vivo* evidence data that IL-1 β blockade attenuates tumor fibrosis, and (4) demonstrating, for the first time to our best knowledge, that high stromal IL-1 β expression is associated with poor overall prognosis. Closely related to our study, mutant *KRAS* sustains NF- κ B activity in PDAC cells through autocrine secretion of IL-1 α , and administration of IL-1R antagonist, Anakinra, potentiates the efficacy of gemcitabine in a murine xenograft model(46–49). As of now, the safety of Anakinra in combination with FOLFIRINOX chemotherapy is being tested in a clinical trial for PDAC patient (NCT02021422). However, we also found that PDAC CM may activate p65 in pancreatic

fibroblasts without phosphorylating IRAK4, suggesting presence of other ligands in the CM that may activate NF- κ B independently of IRAK4.

In summary, our study shed new light on IRAK4 as an important regulator that drives NF- κ B activity, pro-tumorigenic and the fibrogenic function of CAFs. Based on our data and work from others(31, 32), we proposed that tumor-stroma IL-1 β -IRAK4 feedforward circuitry as a key mechanism that drives tumor fibrosis, chemoresistance and poor prognosis in PDAC. Therefore, disrupting this circuitry represents a promising therapeutic strategy that may potentiate chemotherapy in PDAC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Feig C, Gopinathan A, Neesse A, Chan DS, Cook N, Tuveson DA. The pancreas cancer microenvironment. *Clin Cancer Res.* 2012; 18(16):4266–76. [PubMed: 22896693]
2. Mitchem JB, Brennan DJ, Knolhoff BL, Belt BA, Zhu Y, Sanford DE, et al. Targeting Tumor-Infiltrating Macrophages Decreases Tumor-Initiating Cells, Relieves Immunosuppression, and Improves Chemotherapeutic Responses. *Cancer Res.* 2013
3. Goedegebuure P, Mitchem JB, Porembka MR, Tan MC, Belt BA, Wang-Gillam A, et al. Myeloid-derived suppressor cells: general characteristics and relevance to clinical management of pancreatic cancer. *Curr Cancer Drug Targets.* 2011; 11(6):734–51. [PubMed: 21599634]
4. Bayne LJ, Beatty GL, Jhala N, Clark CE, Rhim AD, Stanger BZ, et al. Tumor-derived granulocyte-macrophage colony-stimulating factor regulates myeloid inflammation and T cell immunity in pancreatic cancer. *Cancer cell.* 2012; 21(6):822–35. [PubMed: 22698406]
5. Provenzano PP, Cuevas C, Chang AE, Goel VK, Von Hoff DD, Hingorani SR. Enzymatic targeting of the stroma ablates physical barriers to treatment of pancreatic ductal adenocarcinoma. *Cancer Cell.* 2012; 21(3):418–29. [PubMed: 22439937]
6. DuFort CC, DelGiorno KE, Hingorani SR. Mounting Pressure in the Microenvironment: Fluids, Solids, and Cells in Pancreatic Ductal Adenocarcinoma. *Gastroenterology.* 2016; 150(7):1545–57 e2. [PubMed: 27072672]
7. Sangaletti S, Colombo MP. Matricellular proteins at the crossroad of inflammation and cancer. *Cancer Lett.* 2008; 267(2):245–53. [PubMed: 18471960]
8. Olive KP. Stroma, Stroma Everywhere (Far More Than You Think). *Clin Cancer Res.* 2015; 21(15):3366–8. [PubMed: 25979482]
9. Hwang RF, Moore T, Arumugam T, Ramachandran V, Amos KD, Rivera A, et al. Cancer-associated stromal fibroblasts promote pancreatic tumor progression. *Cancer Res.* 2008; 68(3):918–26. [PubMed: 18245495]

10. Jacobetz MA, Chan DS, Neesse A, Bapiro TE, Cook N, Frese KK, et al. Hyaluronan impairs vascular function and drug delivery in a mouse model of pancreatic cancer. *Gut*. 2013; 62(1):112–20. [PubMed: 22466618]
11. Leca J, Martinez S, Lac S, Nigri J, Secq V, Rubis M, et al. Cancer-associated fibroblast-derived annexin A6+ extracellular vesicles support pancreatic cancer aggressiveness. *J Clin Invest*. 2016; 126(11):4140–56. [PubMed: 27701147]
12. Sousa CM, Biancur DE, Wang X, Halbrook CJ, Sherman MH, Zhang L, et al. Pancreatic stellate cells support tumour metabolism through autophagic alanine secretion. *Nature*. 2016; 536(7617):479–83. [PubMed: 27509858]
13. Hingorani SR, Harris WP, Beck JT, Berdov BA, Wagner SA, Pshevlotsky EM, et al. Phase Ib Study of PEGylated Recombinant Human Hyaluronidase and Gemcitabine in Patients with Advanced Pancreatic Cancer. *Clin Cancer Res*. 2016; 22(12):2848–54. [PubMed: 26813359]
14. Kim EJ, Sahai V, Abel EV, Griffith KA, Greenson JK, Takebe N, et al. Pilot clinical trial of hedgehog pathway inhibitor GDC-0449 (vismodegib) in combination with gemcitabine in patients with metastatic pancreatic adenocarcinoma. *Clin Cancer Res*. 2014; 20(23):5937–45. [PubMed: 25278454]
15. Hurwitz HI, Uppal N, Wagner SA, Bendell JC, Beck JT, Wade SM 3rd, et al. Randomized, Double-Blind, Phase II Study of Ruxolitinib or Placebo in Combination With Capecitabine in Patients With Metastatic Pancreatic Cancer for Whom Therapy With Gemcitabine Has Failed. *J Clin Oncol*. 2015; 33(34):4039–47. [PubMed: 26351344]
16. Rhim AD, Oberstein PE, Thomas DH, Mirek ET, Palermo CF, Sastra SA, et al. Stromal elements act to restrain, rather than support, pancreatic ductal adenocarcinoma. *Cancer Cell*. 2014; 25(6):735–47. [PubMed: 24856585]
17. Ozdemir BC, Pentcheva-Hoang T, Carstens JL, Zheng X, Wu CC, Simpson TR, et al. Depletion of carcinoma-associated fibroblasts and fibrosis induces immunosuppression and accelerates pancreas cancer with reduced survival. *Cancer Cell*. 2014; 25(6):719–34. [PubMed: 24856586]
18. Zhang D, Li L, Jiang H, Knolhoff BL, Lockhart AC, Wang-Gillam A, et al. Constitutive IRAK4 Activation Underlies Poor Prognosis and Chemoresistance in Pancreatic Ductal Adenocarcinoma. *Clin Cancer Res*. 2017; 23(7):1748–59. [PubMed: 27702822]
19. Erez N, Truitt M, Olson P, Arron ST, Hanahan D. Cancer-Associated Fibroblasts Are Activated in Incipient Neoplasia to Orchestrate Tumor-Promoting Inflammation in an NF-kappaB-Dependent Manner. *Cancer Cell*. 2010; 17(2):135–47. [PubMed: 20138012]
20. Pallangyo CK, Ziegler PK, Greten FR. IKKbeta acts as a tumor suppressor in cancer-associated fibroblasts during intestinal tumorigenesis. *J Exp Med*. 2015; 212(13):2253–66. [PubMed: 26621452]
21. Koliarakis V, Pasparakis M, Kollias G. IKKbeta in intestinal mesenchymal cells promotes initiation of colitis-associated cancer. *J Exp Med*. 2015; 212(13):2235–51. [PubMed: 26621453]
22. Ochi A, Nguyen AH, Bedrosian AS, Mushlin HM, Zarbakhsh S, Barilla R, et al. MyD88 inhibition amplifies dendritic cell capacity to promote pancreatic carcinogenesis via Th2 cells. *J Exp Med*. 2012; 209(9):1671–87. [PubMed: 22908323]
23. Bachem MG, Schneider E, Gross H, Weidenbach H, Schmid RM, Menke A, et al. Identification, culture, and characterization of pancreatic stellate cells in rats and humans. *Gastroenterology*. 1998; 115(2):421–32. [PubMed: 9679048]
24. Jiang H, Hegde S, Knolhoff BL, Zhu Y, Herndon JM, Meyer MA, et al. Targeting focal adhesion kinase renders pancreatic cancers responsive to checkpoint immunotherapy. *Nat Med*. 2016; 22(8):851–60. [PubMed: 27376576]
25. Lim KH, Langley E, Gao F, Luo J, Li L, Meyer G, et al. A clinically feasible multiplex proteomic immunoassay as a novel functional diagnostic for pancreatic ductal adenocarcinoma. *Oncotarget*. 2017; 8(15):24250–61. [PubMed: 28445954]
26. Suzuki N, Suzuki S, Duncan GS, Millar DG, Wada T, Mirtsos C, et al. Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. *Nature*. 2002; 416(6882):750–6. [PubMed: 11923871]
27. Kondo M, Tahara A, Hayashi K, Abe M, Inami H, Ishikawa T, et al. Renoprotective effects of novel interleukin-1 receptor-associated kinase 4 inhibitor AS2444697 through anti-inflammatory

- action in 5/6 nephrectomized rats. *Naunyn Schmiedebergs Arch Pharmacol.* 2014; 387(10):909–19. [PubMed: 25052043]
28. Ohlund D, Elyada E, Tuveson D. Fibroblast heterogeneity in the cancer wound. *J Exp Med.* 2014; 211(8):1503–23. [PubMed: 25071162]
 29. Li S, Strelow A, Fontana EJ, Wesche H. IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase. *Proc Natl Acad Sci U S A.* 2002; 99(8):5567–72. [PubMed: 11960013]
 30. Kiefel H, Bondong S, Erbe-Hoffmann N, Hazin J, Riedle S, Wolf J, et al. L1CAM-integrin interaction induces constitutive NF-kappaB activation in pancreatic adenocarcinoma cells by enhancing IL-1beta expression. *Oncogene.* 2010; 29(34):4766–78. [PubMed: 20543863]
 31. Muerkoster S, Wegehenkel K, Arlt A, Witt M, Sipos B, Kruse ML, et al. Tumor stroma interactions induce chemoresistance in pancreatic ductal carcinoma cells involving increased secretion and paracrine effects of nitric oxide and interleukin-1beta. *Cancer Res.* 2004; 64(4):1331–7. [PubMed: 14973050]
 32. Arlt A, Vorndamm J, Muerkoster S, Yu H, Schmidt WE, Folsch UR, et al. Autocrine production of interleukin 1beta confers constitutive nuclear factor kappaB activity and chemoresistance in pancreatic carcinoma cell lines. *Cancer Res.* 2002; 62(3):910–6. [PubMed: 11830551]
 33. Schmid MC, Avraamides CJ, Foubert P, Shaked Y, Kang SW, Kerbel RS, et al. Combined blockade of integrin-alpha4beta1 plus cytokines SDF-1alpha or IL-1beta potently inhibits tumor inflammation and growth. *Cancer Res.* 2011; 71(22):6965–75. [PubMed: 21948958]
 34. Incio J, Liu H, Suboj P, Chin SM, Chen IX, Pinter M, et al. Obesity-Induced Inflammation and Desmoplasia Promote Pancreatic Cancer Progression and Resistance to Chemotherapy. *Cancer discovery.* 2016; 6(8):852–69. [PubMed: 27246539]
 35. Richards KE, Zeleniak AE, Fishel ML, Wu J, Littlepage LE, Hill R. Cancer-associated fibroblast exosomes regulate survival and proliferation of pancreatic cancer cells. *Oncogene.* 2017; 36(13):1770–8. [PubMed: 27669441]
 36. Carbone C, Melisi D. NF-kappaB as a target for pancreatic cancer therapy. *Expert opinion on therapeutic targets.* 2012; 16(Suppl 2):S1–10.
 37. Suzuki N, Suzuki S, Yeh WC. IRAK-4 as the central TIR signaling mediator in innate immunity. *Trends Immunol.* 2002; 23(10):503–6. [PubMed: 12297423]
 38. Teague A, Lim KH, Wang-Gillam A. Advanced pancreatic adenocarcinoma: a review of current treatment strategies and developing therapies. *Ther Adv Med Oncol.* 2015; 7(2):68–84. [PubMed: 25755680]
 39. Neesse A, Algul H, Tuveson DA, Gress TM. Stromal biology and therapy in pancreatic cancer: a changing paradigm. *Gut.* 2015; 64(9):1476–84. [PubMed: 25994217]
 40. von Ahrens D, Bhagat TD, Nagrath D, Maitra A, Verma A. The role of stromal cancer-associated fibroblasts in pancreatic cancer. *J Hematol Oncol.* 2017; 10(1):76. [PubMed: 28351381]
 41. Fang H, Declerck YA. Targeting the tumor microenvironment: from understanding pathways to effective clinical trials. *Cancer Res.* 2013; 73(16):4965–77. [PubMed: 23913938]
 42. DeClerck YA, Pienta KJ, Woodhouse EC, Singer DS, Mohla S. The Tumor Microenvironment at a Turning Point Knowledge Gained Over the Last Decade, and Challenges and Opportunities Ahead: A White Paper from the NCI TME Network. *Cancer Res.* 2017; 77(5):1051–9. [PubMed: 28209610]
 43. Zambirinis CP, Levie E, Nguy S, Avanzi A, Barilla R, Xu Y, et al. TLR9 ligation in pancreatic stellate cells promotes tumorigenesis. *J Exp Med.* 2015; 212(12):2077–94. [PubMed: 26481685]
 44. Ochi A, Graffeo CS, Zambirinis CP, Rehman A, Hackman M, Fallon N, et al. Toll-like receptor 7 regulates pancreatic carcinogenesis in mice and humans. *J Clin Invest.* 2012; 122(11):4118–29. [PubMed: 23023703]
 45. Xiao Q, Zhou D, Rucki AA, Williams J, Zhou J, Mo G, et al. Cancer-Associated Fibroblasts in Pancreatic Cancer Are Reprogrammed by Tumor-Induced Alterations in Genomic DNA Methylation. *Cancer Res.* 2016; 76(18):5395–404. [PubMed: 27496707]
 46. Zhuang Z, Ju HQ, Aguilar M, Gocho T, Li H, Iida T, et al. IL1 Receptor Antagonist Inhibits Pancreatic Cancer Growth by Abrogating NF-kappaB Activation. *Clin Cancer Res.* 2016; 22(6):1432–44. [PubMed: 26500238]

47. Ling J, Kang Y, Zhao R, Xia Q, Lee DF, Chang Z, et al. KrasG12D-induced IKK2/beta/NF-kappaB activation by IL-1alpha and p62 feedforward loops is required for development of pancreatic ductal adenocarcinoma. *Cancer Cell*. 2012; 21(1):105–20. [PubMed: 22264792]
48. Melisi D, Niu J, Chang Z, Xia Q, Peng B, Ishiyama S, et al. Secreted interleukin-1alpha induces a metastatic phenotype in pancreatic cancer by sustaining a constitutive activation of nuclear factor-kappaB. *Molecular cancer research : MCR*. 2009; 7(5):624–33. [PubMed: 19435817]
49. Niu J, Li Z, Peng B, Chiao PJ. Identification of an autoregulatory feedback pathway involving interleukin-1alpha in induction of constitutive NF-kappaB activation in pancreatic cancer cells. *J Biol Chem*. 2004; 279(16):16452–62. [PubMed: 14679213]

Figure 1. IRAK4 is constitutively phosphorylated and drives NF- κ B activity in PDAC CAFs
A, Representative IHC images showing p-IRAK4 staining in CAFs in normal pancreas, pancreatitis and PDAC samples from a commercial TMA (US Biomax PA2081). Positive (+) or negative (–) staining was determined by GI pathologist. **B**, **C**, Representative IHC images showing presence of p-IRAK4 staining in CAFs of Capan-1 xenograft and murine KPC tumors. **D**, Confocal IF images of human PDAC, Capan-1 xenograft and KPC tumors showing p-IRAK4 staining in α -SMA⁺ in CAFs. (**A–D**, scale bars: 50 μ m). **E**, Western blots showing higher p-IRAK, p-p65 levels in four PDAC CAF lines specified by α -SMA expression, as opposed to normal dermal (hFB) and pancreatic (SC00A5) fibroblasts. **F**,

Western blots showing dose-dependent suppression of p-IRAK4 and p-p65 levels in CAF-2 and CAF-8 following overnight treatment with AS2444697 or IRAK1/4 inhibitor at the indicated doses. **G, H**, Western blots showing changes in p-p65 levels following: **(G)** ectopic expression of empty vector, wild-type, or kinase-dead IRAK4 in two normal fibroblasts (hFB and SC00A5); **(H)** knockdown of IRAK4 using two shRNAs in CAF-2 and CAF-8.

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Figure 2. CAF IRAK4 supports PDAC cell proliferation, migration and tumorigenesis

A, Co-culture assay showing abundance of MIA Paca-2 cells stably expressing luciferase/RFP, as determined by luciferase titer and RFP⁺ cells (Suppl. Fig. 2A), when cultured alone or at 1:1 ratio with GFP⁺ CAF-2 or CAF-8 cells expressing scramble shRNA or two different shIRAK4s. **B**, Wound-healing and **C**, transwell migration assay of MIA Paca-2 cells cultured in CM collected from SC00A5, CAF-2/8 cells expressing scramble shRNA or different shIRAK4. **D**, Final weights and picture of MIA Paca-2 tumors injected alone or in 1:2 ratio with the indicated CAFs (N=6 mice/group). **E**, Quantification and representative co-IF images of dual pan-cytokeratin⁺ and Ki-67⁺ cells in MIA Paca-2 tumors from experiment Fig. 2D. All *in vitro* experiments were conducted at least three times in triplicates and represented as mean ± SEM (*p<0.05, **p<0.01, ***p<0.001).

Figure 3. CAF IRAK4 contributes to PDAC tumor fibrosis

A, Quantification and representative images of gel contraction assay showing dose-dependent effect of IRAK4 inhibitor AS2444697 on the ability of CAF-2 and CAF-8 to contract type 1 collagen *in vitro*. **B**, Quantitative PCR of collagen 1 mRNA in the indicated fibroblast lines. Data (means $\pm$ SEM) represents one of three experiments done in biological duplicates and technical triplicates. **C**, Quantification and representative images (200X) of Sirius Red⁺ area in tumors arising from MIA Paca-2 injected alone or at 1:2 ratio with the indicated CAFs (N=6/group). **D**, Final weights of tumors arising from KPC2 cells injected subcutaneously in wild-type C57BL/6J mice, *IRAK4*-null littermates, alone or in

combination with wild-type or *IRAK4*-null MEFs (N=6–10/group). **E, F**, Quantification and representative images of (E) dual pan-cytokeratin⁺ and Ki-67⁺ cells, and (F) Sirius Red⁺ area in the indicated KPC2 tumors. (6–8 tumors/group, ten 20X fields were analyzed per tumor). Data represents combined means $\pm$ SEM. (*p<0.05, **p<0.01, ***p<0.001).

Figure 4. Autocrine IL-1 β activates IRAK4 in CAFs

A, Cytokine array showing altered abundance of chemokines/cytokines in serum-free CM collected from CAF-2 cells stably expressing scramble or IRAK4 shRNAs. **B**, Quantitative PCR showing fold changes in mRNA levels of the indicated genes in CAF-2 expressing scramble shRNA treated with DMSO, IRAK1/4 inhibitor or AS2444697 overnight, or stably expressing two different shIRAK4s. Data (means $\pm$ SEM) represents one of two experiments done in biological duplicates and technical triplicates. **C**, Consecutive IHC sections showing expression of IL-1 β in α -SMA+ CAFs in two representative human PDAC samples from TMA (US Biomax PA 2081). Of all 117 PDAC samples, 96 showed positive, and 21 showed

negative IL-1 β IHC staining in α -SMA⁺ CAFs. **D**, Confocal IF images showing presence of IL-1 β in α -SMA⁺ CAFs in two human surgical PDAC and murine KPC tumors. **E**, ELISA assay showing abundance of IL-1 β protein in CM collected from the indicated cells treated with DMSO or AS2444697 overnight (left panel), or from CAF-2 cells stably expressing scramble shRNA or two different shIRAK4s (right panel). Experiments were done twice in triplicates. Data presented as means $\pm$ SEM (* p <0.05, ** p <0.01 by two-tailed t test). **F**, Western blots showing dose-dependent suppression of p-IRAK4 and p-p65 levels in CAF-2 treated overnight with nIL-1 β antibody or Anakinra.

Figure 5. PDAC cells secrete IL-1 β to activate CAF IRAK4

A, Western blots showing changes in p-IRAK4 and p-p65 levels of SC00A5 cells incubated overnight with serum-free DMEM alone or at 1:1 ratio with serum-free CM collected from the indicated PDAC lines. **B**, IL-1 β ELISA of CM collected from the indicated PDAC lines used in Fig. 5A. Experiment was done twice in triplicates and data represented as means $\pm$ SEM. **C**, Quantitative PCR of IL-1 β mRNA in the indicated fibroblast lines incubated with or without serum-free MIA Paca-2 CM overnight. **D**, Venn diagrams of genes expressed at 1.5-fold difference FDR < 0.1 in DMSO vs. IRAK1/4 inhibitor treatment in Capan-1 and PANC-1 cells. Expression values used were gene-wise z-score normalized. **E**, Western blots

showing changes in p-IRAK4 and p-p65 levels of SC00A5 cells incubated with serum-free DMEM, MIA Paca-2 or Capan-1 CM in the absence or presence of neutralizing anti-human IL-1 β antibody. **F**, Gel contraction assay showing a role of IL-1 β in MIA Paca-2 CM in inducing collagen contraction of SC00A5 cells. Experiment was done three time in triplicates and presented as means $\pm$ SEM. **G**, Western blots confirming IL-1R protein knockdown in SC00A5 cells stably expressing two different shIL-1R (left panel). These three SC00A5 lines were then treated with serum-free DMEM or MIA Paca-2 CM overnight, followed by western blots showing changes in p-IRAK4 and p-p65 levels. (*p<0.05, **p<0.01, ***p<0.001).

Figure 6. CAF IRAK4 drives IL-1 β secretion to promote PDAC cell NF- κ B activity and survival

A, Alamar blue assay showing viability of the indicated PDAC lines cultured in serum-free medium alone or at 1:1 ratio with different CAF CM for 72 hours. **B**, NF- κ B luciferase reporter assay of MIA PaCa-2 cells incubated without or with different CAF CM overnight. **C**, Alamar blue assay showing gemcitabine dose-response inhibition of MIA PaCa-2 and CFPAC-1 cells cultured with CM collected from SC00A5, or CAF-2 cells expressing scramble shRNA or two different shIRAK4s for 72 hours. Changes in IC₅₀ of gemcitabine in each condition were shown. **D**, Caspase 3/7 luciferase reporter activity in MIA PaCa-2 cells cultured in the indicated CAF CM, treated with vehicle (PBS) or gemcitabine for 24 hours. **E**, Alamar blue assay showing viability of two PDAC lines cultured in serum free medium alone or CAF-2 CM for 72 hours in the absence or presence of neutralizing anti-human IL-1 β antibody. **F**, NF- κ B luciferase reporter assay of MIA PaCa-2 cells treated with anti-IL-1 β neutralizing antibody without or with CAF-2 CM overnight. All experiments were done three times in triplicates and data presented as means $\pm$ SEM. (* p <0.05, ** p <0.01, *** p <0.001).

Figure 7. Disruption of tumor-stroma IL-1 β -IRAK4 circuitry attenuates tumor fibrosis and augments the effect of gemcitabine *in vivo*

A, Final weights and representative pictures of Capan-1 tumors injected subcutaneously alone, or in 1:2 ratio with CAF-2 expressing scramble shRNA, shIRAK4 or shIL-1R. (N=8/group). **B**, **C**, Quantification of (B) proliferating (dual pan-cytokeratin⁺ and Ki-67⁺ cells) or (C) α -SMA⁺ cells in the indicated Capan-1 tumors. **D**, Final weights and representative pictures of KPC2 tumors grown in mice treated with vehicle (V), anti-mouse IL-1 β neutralizing antibody (nIL-1 β), gemcitabine (GEM), AS2444697, or in dual combinations. (N=10–14/group combined from two independent experiments). **E**, **F**, **G**, Quantification of

proliferating (dual pan-cytokeratin⁺ and Ki-67⁺ cells), apoptotic cells (cleaved caspase-3⁺), or degree of fibrosis (Sirius Red⁺ area) of KPC2 tumors treated as indicated (N=10/group). **H**, (Left) Representative IHC images showing assigned intensity scores (0=absence/faint, 1=weak, 2=moderate, 3=strong) of stromal IL-1 β staining in a PDAC tissue microarray (N=125). (Right) Kaplan-Meier survival analysis and median survival of 125 patients based on low (0–1) or high (2–3) stromal IL-1 β IHC score. **I**, Schematics depicting the proposed model in which PDAC cells and CAFs conspire to establish a feedforward IL-1 β –IRAK4 circuitry that leads to tumor fibrosis, chemoresistance and poor prognosis.