Interleukin-6 Is Required for Pancreatic Cancer Progression by Promoting MAPK Signaling Activation and Oxidative Stress Resistance

Yaqing Zhang1, Wei Yan2,5, Meredith A. Collins6, Filip Bednar1, Sabita Rakshit1, Bruce R. Zetter8, Ben Z. Stanger9,10, Ivy Chung11,12, Andrew D. Rhim4, and Marina Pasca di Magliano1,3,7

Abstract

Pancreatic cancer, one of the deadliest human malignancies, is almost invariably associated with the presence of an oncogenic form of Kras. Mice expressing oncogenic Kras in the pancreas recapitulate the stepwise progression of the human disease. The inflammatory cytokine interleukin (IL)-6 is often expressed by multiple cell types within the tumor microenvironment. Here, we show that IL-6 is required for the maintenance and progression of pancreatic cancer precursor lesions. In fact, the lack of IL-6 completely ablates cancer progression even in presence of oncogenic Kras. Mechanistically, we show that IL-6 synergizes with oncogenic Kras to activate the reactive oxygen species detoxification program downstream of the mitogen-activated protein kinase/extracellular signal—regulated kinase (MAPK/ERK) signaling cascade. In addition, IL-6 regulates the inflammatory microenvironment of pancreatic cancer throughout its progression, providing several signals that are essential for carcinogenesis. Thus, IL-6 emerges as a key player at all stages of pancreatic carcinogenesis and a potential therapeutic target. Cancer Res; 73(20); 6359–74. ©2013 AACR.

Introduction

Pancreatic cancer is one of the deadliest human malignancies, with a 5-year survival rate of less than 6% (1–3). The dismal survival rate has remained essentially unchanged over the course of the past 40 years, highlighting the need for a deeper understanding of the biology of this disease, which might lead to new targeting strategies. Recent sequencing studies (4) have confirmed the decades old observation that KRAS is the most commonly mutated gene in pancreatic cancer (5, 6). KRAS mutations occur early during disease progression, in pancreatic cancer precursor lesions known as pancreatic intraepithelial neoplasias (PanIN; ref. 7). However, KRAS mutations are found in healthy pancreata at a much higher rate than the incidence of pancreatic cancer (8, 9), suggesting that additional genetic, epigenetic, or environmental factors are required for tumorigenesis.

Chronic pancreatitis confers a significantly increased lifetime risk to develop pancreatic cancer, and is thus one of the highest known risk factors (10). The relationship between acute pancreatitis and carcinogenesis is less well established; however, acute pancreatitis can progress to chronic pancreatitis in individuals carrying additional risk factors (such as smoking or alcohol abuse; ref. 11). In mice, both chronic and acute pancreatitis synergize with the presence of oncogenic Kras to drive formation of PanINs (12, 13). The cytokine interleukin (IL)-6 is upregulated during pancreatitis in mice and humans (14). IL-6 plays an essential procarcinogenic function in colon and liver cancer (15, 16). In contrast, at least in mice, its role is secondary to the closely related cytokine IL-11 in gastric cancer (17). Thus, the relevance of IL-6 in carcinogenesis is tissue-specific. Previous studies have identified IL-6 and its downstream effector Stat3 as being important for pancreatic cancer initiation in mouse models of this disease (18–20). However, whether IL-6 plays a role in inflammation-driven pancreatic carcinogenesis, as well as its role at later stages of carcinogenesis, was not known. These questions have therapeutic relevance, as patients with pancreatitis are a population where preventive strategies could be successfully used to avoid progression to cancer. Preventive strategies that
block PanIN progression to cancer could conceivably also be useful in familial pancreatic cancer, as well as to prevent recurrence in patients who have undergone resection of the primary tumor.

In this study, we set out to determine whether sustained IL-6 expression was required to initiate pancreatitis-associated pancreatic cancer. We used a genetically engineered mouse model of pancreatic cancer, the iKras\(^{m2} \) mouse, based on pancreas-specific, inducible, and reversible expression of oncogenic Kras\(^{G12D} \) (Kras\(^{m2} \)) recently described by our group (21). This model develops pancreatic cancer in a stepwise manner within an intact microenvironment. Our data show that IL-6 was dispensable for the initiation of pancreatic cancer precursor lesions in the presence of inflammation. However, we uncovered a previously unrecognized role for IL-6 in the maintenance of these precursor lesions and progression to cancer. Thus, our data set the rationale for exploring IL-6 as a therapeutic target in pancreatic cancer.

Materials and Methods

Mouse strains

We generated iKras\(^{m2} \)IL-6\(^{-/-} \) mice by crossing previously described triple-transgenic mice iKras\(^{m2} \) (p14-CreB26-nTR-ires-EGFP;TetO-Kras\(^{G12D} \); ref. 21) with IL-6-deficient mice (B6.Cg-Il6tm1Kopf/J, The Jackson Laboratory). Combinations of single- or double-mutant littermates were used as controls. Animals were housed in specific pathogen-free facilities of the University of Michigan Comprehensive Cancer Center (Ann Arbor, MI). Studies were carried out in compliance with University of Michigan University Committee on Use and Care of Animals guidelines. Pdx1-Cre;Kras\(^{LSL-G12D/+; p53fl/fl; }\) (KPCY) mice (22) were housed in a specific pathogen-free facility at the University of Pennsylvania (Philadelphia, PA) and in compliance with Penn Institutional Animal Care and Use Committee guidelines.

Doxycycline treatment

iKras\(^{m2} \) or iKras\(^{m2} \)IL-6\(^{-/-} \) mice were treated with doxycycline to induce Kras\(^{G12D} \) expression. Doxycycline was administered in the low-dose doxycycline chow (50 mg/kg) or drinking water, at a concentration of 0.2 g/L in a solution of 3% sucrose, and replaced every 3 to 4 days.

Primary tumor cells

Primary tumor specimen implantation and preparation of single-cell suspensions of tumor cells were carried out as previously described (23). All samples derived from human subjects were approved by University of Michigan and the Federal Institutional Review Board. Establishment of primary mouse pancreatic cancer cell line from iKras\(^{m2} \)p53\(^{-/-} \) mouse tumor was previously described (24).

Histopathologic and histologic analysis

The histopathologic analysis was conducted as previously described (21). The data were expressed as percentage of total counted clusters. Error bars represent SE.

Flow cytometry

Single-cell suspensions of fresh spleen and pancreas were prepared as follows: spleens were crushed and passed through a 40-μm cell strainer, washed once with RPMI/10% fetal calf serum (FCS), and treated with BCA lysis buffer (eBioscience) to eliminate BCA. Pancreata were minced using sterile scalpels, then incubated in 1 mg/mL collagenase (Sigma-Aldrich) in Hank’s Balanced Salt Solution (HBSS) for 15 minutes at 37°C before passing through a 40-μm cell strainer. Single-cell suspensions were stained in HBSS/2% FCS with the following antibodies: CD3 (17A2), CD4 (RM4-5), CD8α (53-6.7), CD25 (PC61), CD11b (M1/70), F4/80 (BM8), CD11c (HL3), Gr-1 (RB6-8C5), Foxp3 (FJK-16s; all from BD Pharmingen), and CD45 (MCD4530; Invitrogen). Flow cytometry was conducted using a CyAn ADP Analyzer (Beckman Coulter), and data were analyzed with Summit 4.3 software.

Reactive oxygen species induction and detection

Primary mouse pancreatic cancer cell line 9805 was treated with 600 μmol/L H₂O₂ for 1 hour and the presence of reactive oxygen species (ROS) was detected using CellROX Green reagent (C10444; Invitrogen), a fluorogenic probe, according to the manufacturer’s instructions. Briefly, cells were then incubated with 10 μmol/L CellROX Green in RPMI with 10% FBS for 30 minutes at 37°C. Cells were then washed in PBS and imaged on a Olympus IX71 inverted microscope. Signal intensity was analyzed using Image-Pro Plus software.

In vivo anti-IL-6 treatment

KPCY mice aged 10 weeks were randomized to two treatment arms: anti-IL-6 antibody treatment (25 mg/kg; clone MP5-20F3; BioXCell) or rat immunoglobulin G1 (IgG1) control (25 mg/kg; BE0088; BioXCell). Mice (3 for each group) received treatments on days 0, 2, 4, and 6 intraperitoneally and were then sacrificed on day 7 for histologic analysis. PanIN lesions in three sections per mouse were quantified by grade in a blinded manner. Data are expressed as number of acinar-ductal metaplasias (ADM) or PanINs of each grade per medium (10 ×) powered field. No discernible side effects were noted in either of the treatment groups.

Statistical analysis

All data were presented as mean ± SEM. Intergroup comparisons were conducted using the Student t test. Prism 6 was used for all statistical analyses, and P < 0.05 was considered statistically significant.

Detailed procedures and standard procedures are included in the Supplementary Methods; detailed antibody information and primer sequences are listed in Supplementary Tables S1 and S2.

Results

IL-6 is expressed by several cell types within the pancreatic cancer microenvironment

Analysis of human and mouse pancreatic cancer samples revealed IL-6 immunostaining in several cell compartments, including epithelial cells, smooth muscle actin (SMA)–positive fibroblasts, and immune cells (Supplementary Fig. S1A and
S1B). Interestingly, IL-6 was expressed in mouse primary pancreatic fibroblasts only upon incubation with conditioned medium from pancreatic cancer cells (Supplementary Fig. S1C). Because previous studies had described expression restricted to immune cells (18), we sought to confirm the immunostaining results by quantitative real-time PCR (qRT-PCR), and observed that primary mouse pancreatic fibroblasts in culture expressed Il6 mRNA when exposed to conditioned medium from pancreatic cancer cells (Supplementary Fig. S1D). Moreover, one of two primary pancreatic cancer cell lines tested expressed IL-6 (Supplementary Fig. S1E). Thus, multiple sources of IL-6 are present within the pancreatic cancer microenvironment.

**IL-6 is required for PanIN formation in iKras mice with embryonic Kras activation**

In iKras<sup>+</sup> mice, the expression of oncogenic Kras can be timed at will by adding or removing doxycycline from the animal’s food or water (21). We previously reported that activation of Kras<sup>G12D</sup> in adult animals leads to PanIN formation with low penetrance and long latency (21). In contrast, embryonic activation of Kras<sup>G12D</sup> (Supplementary Fig. S1F) resulted in PanIN formation in all the animals by 6 weeks of age (Supplementary Fig. S1I). To determine the effect of IL-6 inactivation on PanIN formation in iKras<sup>+</sup> mice, we generated iKras<sup>fl/fl</sup> mice with IL-6 deficiency (IL-6<sup>−/−</sup> mice) (Fig. 1A). iKras<sup>−/−</sup> and iKras<sup>fl/fl</sup> littermates were sacrificed at 6 weeks of age and their pancreata were harvested (Supplementary Fig. S1F). The expression of the Kras<sup>G12D</sup> transgene was comparable in iKras<sup>−/−</sup> and iKras<sup>fl/fl</sup>; IL-6<sup>−/−</sup> mice, and undetectable in wild-type controls. Il6 mRNA was elevated in iKras<sup>+</sup> pancreata compared with control and, as expected, undetectable in iKras<sup>−/−</sup> mice (Supplementary Fig. S1G). Histologic examination of iKras<sup>+</sup> pancreata revealed PanIN lesions surrounded by extensive fibro-inflammatory stroma (Supplementary Fig. S1I). We detected IL-6 expression, as well as activation of the downstream effector Stat3, both in the lesions and in the surrounding stroma. Moreover, we detected elevated expression of phosphorylated extracellular signal–regulated kinase 1/2 (p-ERK1/2), as readout of mitogen-activated protein kinase (MAPK) pathway activity. Similar findings were obtained in iKras<sup>−/−</sup> mice. In iKras<sup>−/−</sup> pancreata, we observed a majority of normal acini with infrequent areas of ADM and rare PanINs (Supplementary Fig. S1I; and histopathologic analysis in Supplementary Fig. S1H). As expected, IL-6 expression was completely abrogated in these tissues. Interestingly, the residual lesions in iKras<sup>−/−</sup> mice had reduced p-ERK1/2 and p-Stat3 compared with lesions in iKras<sup>+</sup> mice. Thus, in the iKras model, IL-6 is important for the onset of PanINs. These findings are consistent with the observation that IL-6 is important for pancreatic cancer initiation (18).

**Pancreatitis-driven PanINs in IL-6–deficient mice have altered signaling and reduced proliferation**

In a next set of experiments, we let the iKras<sup>+</sup> and iKras<sup>−/−</sup> mice reach adulthood in absence of doxycycline, thus maintaining Kras<sup>+</sup> expression off. Doxycycline was then administered when the animals reached 4 to 6 weeks of age, to induce Kras<sup>+</sup> expression, and pancreatitis was induced by two series of caerulein injections, for 2 consecutive days, starting 72 hours after doxycycline administration, as previously described (Fig. 1B; refs. 12, 21). One day later, pancreatitis was evident in control, iKras<sup>+</sup> and iKras<sup>−/−</sup> pancreata, with characteristic acinar damage, ADM, edema, and inflammatory infiltrates, as well as increased expression of p-ERK1/2 and p-Stat3 (Fig. 1C). Flow cytometry showed that the number of infiltrating CD45<sup>+</sup> immune cells was similar in all the experimental groups; although we observed a trend toward reduced infiltration of T cells and a significant increase in macrophages (but not in the related myeloid-derived suppressor cells) in iKras<sup>−/−</sup> mice (Supplementary Fig. S2A). The acute response to pancreatitis is accompanied by upregulation of the MAPK pathway (25, 26), the PI3K/Akt pathway (27), and by activation of Stat3 (19). In iKras<sup>−/−</sup> mice, the levels of p-ERK1/2, p-Stat3 and, to a lesser extent, p-AKT were reduced (Fig. 1D). Thus, while the absence of IL-6 did not prevent induction of pancreatitis, it qualitatively changed the response to the inflammatory stimulus.

To determine whether IL-6 deficiency altered PanIN formation, we dissected tissues 3 weeks post-pancreatitis, when full recovery is expected in control animals, and extensive PanIN lesions are expected in iKras<sup>+</sup> mice (21). Upon histopathologic analysis, both in iKras<sup>+</sup> and in iKras<sup>−/−</sup> mice, we observed pancreas-wide PanIN lesions with accumulation of fibro-inflammatory stroma, characteristic periodic acid-Schiff (PAS) staining, and expression of the PanIN marker Claudin 18 (Fig. 1E). Quantification of the type and extent of lesions revealed no significant changes in iKras<sup>−/−</sup> compared with iKras<sup>+</sup> mice (Fig. 2E; 3 week time point). Thus, PanIN lesions formed even in absence of IL-6, upon induction of pancreatitis.

Further characterization of the lesions in iKras<sup>+</sup> and iKras<sup>−/−</sup> animals revealed striking differences. First, the proliferation index was dramatically reduced in absence of IL-6, both within the lesions and in the surrounding stroma (Fig. 1E; Ki67 immunostaining). Second, the levels of p-ERK1/2, p-Akt, and p-Stat3 were reduced in IL-6–deficient pancreata (Fig. 1F).

In mice bearing mutant Kras<sup>+</sup>, PanIN formation is associated with persistence of inflammatory infiltrates (28). To determine whether the absence of IL-6 altered the prevalence, or nature of the inflammatory infiltrates, we conducted flow cytometry for components of both the innate and adaptive immune systems in iKras<sup>+</sup> and iKras<sup>−/−</sup> pancreata. Our analysis revealed a higher number of inflammatory cells in iKras<sup>+</sup> pancreata compared with the control 3 weeks post-pancreatitis—consistent with the completed repair of the pancreatic tissue in control mice and with the accumulation of a fibro-inflammatory stroma in iKras<sup>+</sup> animals. When we compared iKras<sup>+</sup> with iKras<sup>−/−</sup> pancreata, we did not observe a change in the total immune component, or in the number and nature of most infiltrating T-cell subsets (Supplementary Fig. S2A). However, we observed a reduction in several immune subtypes that have been associated with tumor progression, such as macrophages, myeloid-derived...
Figure 1. IL-6 expression is dispensable for pancreatitis-induced PanIN formation. A, genetic makeup of the iKras<sup>−/−</sup>IL-6<sup>−/−</sup> mouse model. B, experimental design; n = 4–7. C, hematoxylin and eosin (H&E) and immunohistochemistry staining for p-ERK1/2 and p-Stat3 in control, iKras<sup>−/−</sup>, and iKras<sup>−/−</sup>IL-6<sup>−/−</sup> mice pancreata 1 day post-pancreatitis induction. (Continued on the following page.)
monocyte suppressor cells, and a trend toward a reduction in the number of other myeloid-derived suppressor cell subsets and regulatory T cells (Supplementary Fig. S2A). We also observed a dramatic decrease in infiltrating mast cells (Supplementary Fig. S2B), a cell type that has been linked to pancreatic carcinogenesis (29); however, whether the lack of mast cells was the cause or effect of the lack of progression remains to be established.

Taken together, our data showed that pancreatitis-driven PanIN formation was independent of IL-6, possibly mediated by other cytokines that are released during the induction of pancreatitis. However, lesions formed in absence of IL-6 were qualitatively distinct. In fact, IL-6 was required for the activation of the MAPK signaling pathway, and, to a lesser extent, for the activation of Akt and Stat3. Moreover, IL-6–deficient PanINs had a low proliferation index. Thus, while histologically similar, PanINs in iKras and iKras;IL-6−/− mice had significant molecular differences in the activation of signaling pathways underlying pancreatic cancer progression.

IL-6 is necessary for PanIN maintenance and progression

We next compared the kinetics of PanIN progression between iKras+ and iKras+;IL-6−/− mice. We aged cohorts of each genotype (n = 4–7 genotype/time point) for 3, 5, 8, or 17 weeks following induction of pancreatitis (see scheme in Fig. 2A). As previously described, at the 3 weeks time point, histopathologic analysis revealed no significant differences between iKras+ and iKras+;IL-6−/− tissues. Over time, however, iKras+ mice developed high-grade PanIN lesions with sustained MAPK activity (Fig. 2B). We observed PAS-positive epithelial cells and expansion of a collagen-rich stroma (Fig. 2C). In contrast, in iKras+;IL-6−/− tissues, we observed a progressive decrease in the prevalence of PanINs and stroma and conversely an increase of acinar clusters (Fig. 2B and C). PanIN lesions are characterized by distinct microscopic features that can be highlighted by transmission electron microscopy (TEM): multilobular nuclei, lack of large secretory granules (which are found in acinar cell), microvilli on the luminal surface, and large, irregular ductal lumen (Fig. 2D). PanINs in iKras+;IL-6−/− samples had similar features, although the nuclear shape was less irregular. In addition, by 5 weeks after the induction of pancreatitis, apoptotic cells were detected within the ductal structures (Fig. 2D). Within the residual lesions, MAPK activation was low, as determined by p-ERK1/2 immunostaining, and was confined to a small subset of cells (Fig. 2B, insets). In addition, we observed reduction in p-Stat3 and p-Akt levels and, as expected, lack of IL-6 expression (Supplementary Fig. S3A–S3C). By 17 weeks, the pancreata of iKras+;IL-6−/− mice were populated by normal acini for more than 80% of the tissue, with rare ADM and sporadic PanIN1A, as confirmed by histopathologic analysis of de-identified samples (Fig. 2E). Because no lesions were observed at this time point, it was not surprising to observe limited p-ERK1/2 and p-Akt staining; occasional p-Stat3 was observed in acini and ducts of normal appearance, possibly indicating a residual inflammatory response in the pancreas (Supplementary Fig. S3C). Contemporary to the changes in the epithelium, the stroma surrounding the PanIN lesions also underwent major remodeling, resulting in very little residual fibrosis and conversely in accumulation of adipose tissue (Fig. 2B and C). This process correlated with the upregulation of MMP7, MT1-MMP and, to a lesser extent, MMP9 (Supplementary Fig. S4A). These proteases might play a role in the remodeling of the tissue, but future experimental work, beyond the scope of the current study, will be needed to address this possibility. Interestingly, the expression of SMA, a fibroblast activation marker, decreased before the remodeling of the stroma in iKras+;IL-6−/− mice (Supplementary Fig. S3D). Thus, remodeling of the stroma was preceded by the return of fibroblasts to a nonactive state, a similar finding to what we previously observed upon inactivation of Kras expression in the pancreas (21). We then investigated whether IL-6 influenced the expression of other inflammatory cytokines in tissues harvested 3, 5 to 8, or 17 weeks after pancreatitis. We did not observe changes in several cytokines and inflammation-related genes such as Il1b, Il10, Il11, Il17, Cox2, Tgfa, and Ifnγ (Supplementary Fig. S4B). However, in addition to confirming the lack of Il6 expression, we detected lower levels of Il1β (a proinflammatory cytokine), Slfn4 a myeloid activation marker; ref. 30, and Gm-CSF (a cytokine that is required for pancreatic cancer growth; refs. 31, 32) in iKras+; IL-6−/− mice compared with iKras+ (Fig. 3A).

In summary, while pancreatitis-induced lesions in iKras+ mice progressed over time to high-grade PanINs surrounded by abundant stroma, neoplastic lesions in iKras+;IL-6−/− mice regressed, the stroma was remodeled, eventually resulting in a normal pancreatic parenchyma, albeit with some adipose tissue infiltration. The remodeling process in iKras+;IL-6−/− pancreatea resembled the effect of Kras‘ inactivation in PanIN-bearing iKras+ mice (21), indicating that IL-6 is a key player in Kras‘-driven carcinogenesis.

IL-6 regulates proliferation, survival, and differentiation status of PanIN cells

Because expression of IL-6 was associated with enhanced proliferation of PanIN lesions (Fig. 3B), we quantified the proliferation index for the different cell populations present in the tissues, namely ADM/PanIN cells, components of the stroma and acinar cells. In iKras+ mice, the PanIN cells had a high proliferation index that was maintained over time, and proliferation in the stroma reached a peak at 5 to 8 weeks. Proliferation in both compartments was significantly reduced...
Figure 2. IL-6 is necessary for PanIN progression and maintenance. A, experimental design; n = 4-7. B, hematoxylin and eosin (H&E) and p-ERK1/2 staining (insets) of iKras\(^{-}\) and iKras\(^{-}\) mice pancreata 3, 5 to 8, and 17 weeks following pancreatitis. Scale bar, 50 \(\mu\)m. C, Gomori Trichrome staining and PAS staining (insets) of iKras\(^{-}\) and iKras\(^{-}\) mice pancreata. Scale bar, 50 \(\mu\)m. D, TEM image of iKras\(^{-}\) and iKras\(^{-}\) mice pancreata 5 weeks following pancreatitis. Scale bar, 10 \(\mu\)m. E, pathologic analysis. Data represent mean ± SEM; n = 3-5. The statistical difference between iKras\(^{-}\) and iKras\(^{-}\) mice at the same time point per lesion type was determined by two-sided Student t test. *, P < 0.05; **, P < 0.01; ****, P < 0.0001; #, not significant. Doxy, doxycycline.
Figure 3. IL-6 regulates proliferation and survival of PanIN cells. A, qRT-PCR for Il6, Il1b, GM-CSF, and Slfn4 expression in WT, iKras
to iKras;IL-6/C0/C0 mice pancreata. B, Ki67 immunohistochemistry staining of iKras/C3/C0/C0 mice pancreata 3, 5 to 8, and 17 weeks following pancreatitis. Scale bar, 25 mm. C, TUNEL (red) and coimmunofluorescence staining for CK19 (green), SMA (gray), and 4',6-diamidino-2-phenylindole (DAPI; blue). Scale bar, 25 mm. D and E, Ki67 proliferation index (D) in ADM/PanIN, stroma, and acinar cells and quantification (E) of apoptotic cells in iKras/C3/C3/C3/C0/C0 mice pancreata. Data represent mean ± SEM; n = 3. The statistical difference was determined by two-sided Student t test, comparing the different genotype mice at each time point for each category considered (acini, stroma, and ADM/PanIN). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Doxy, doxycycline.
in iKras"IL-6"/−/− lesions. Interestingly, when we compared the proliferation of the acinar compartment between the two genotypes, we observed a significant increase (P < 0.05) in iKras"IL-6"/−/− compared with iKras" at the 5 to 8 weeks and at the 17 weeks time points (Fig. 3B and D). Taken together, our data indicate that IL-6 promotes proliferation of the metastatic/dysplastic cells, and possibly prevents acinar proliferation, a normal aspect of the tissue recovery following pancreatitis.

In iKras"IL-6"/−/− mice, this decrease in proliferation potentially explains the lack of progression in the lesions, but not the progressive elimination of the lesions. To investigate this aspect, we investigated two possible mechanisms of regression: apoptosis of PanIN cells, as well as redifferentiation of PanIN cells into normal acini. First, we determined that PanIN cells have severely compromised survival in absence of IL-6. Apoptotic cells [as determined by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) or cleaved caspase-3 staining] were occasionally present in iKras" tissues; the overall level of apoptosis was low (less than 1% of the epithelial cells), and it decreased over time (Fig. 3C and E and Supplementary Figs. S4C and S5A). In contrast, iKras"IL-6"/−/− PanINs had elevated levels of apoptosis both within the epithelium and in the surrounding stroma (Fig. 3C and E and Supplementary Figs. S4C and S5A). In addition to the immunostaining analysis, we conducted qRT-PCR to determine the relative ratio between the mediators of apoptosis Bak and Bax and the antiapoptotic genes Bcl-2 and Bcl-x. At the 5- to 8-week time point, when most of the changes within the tissue were taking place, iKras" IL-6"/−/− tissues, compared with iKras" had a higher proapoptotic ratio, for Bak/Bcl-x and for Bax/Bcl-x (Supplementary Fig. S5B). In a second set of experiments, we conducted co-immunofluorescent staining with CK19, a ductal and PanIN marker, and amylase, an acinar marker. In iKras" tissues, as expected, we observed almost exclusive expression of CK19 in epithelial cells (Fig. 4A and B). In iKras"IL-6"/−/− tissues, CK19 was prevalent at 3 weeks post-pancreatitis, but by 5 to 8 weeks post-pancreatitis up to one third of the epithelial cells coexpressed CK19 and amylase, and a significant amylase-positive population was observed (Fig. 4A and B). By 17 weeks, CK19-only cells were rare, whereas amylase-positive cells represented the majority of the epithelial population, and some cells coexpressing CK19 and amylase were still present (Fig. 4A and B). The transcription factor Mist1 is a key regulator of acinar cell differentiation (33), and its expression is lost during PanIN formation. Importantly, inactivation of Mist1 in the context of oncogenic Kras accelerates pancreatic carcinogenesis (34), whereas conversely forced expression of Mist1 prevents Kras-driven carcinogenesis (35). As expected, we did not observe any Mist1 expression in the lesions of iKras" mice (Fig. 4C). Similarly, in iKras"IL-6"/−/− lesions at 3 weeks post-pancreatitis, Mist1 expression was absent. However, by 5 weeks post-pancreatitis, Mist1 was expressed in the residual ductal/PanIN structures, thus confirming their mixed differentiation status (Fig. 4C). At later time points, Mist1 expression was confined to acini and excluded from the ducts, as in the normal pancreas.

Taken together, our data indicate that PanIN elimination is underscored by a combination of apoptotic cell death, redifferentiation of cells toward the acinar lineage, and increased proliferation of acinar cells, mechanisms of pancreas repair that we have previously observed upon inactivation of oncogenic Kras in PanIN-bearing mice (21).

**IL-6 is required for Nrf2 upregulation and oxidative stress resistance**

The onset of PanIN formation is accompanied by the activation of a ROS detoxification program (36). PanINs that form in mice deficient for Nrf2, a key factor in the ROS detoxification pathway, are nonproliferative (36). Expression of Nrf2 has been shown to be activated downstream of oncogenic Kras (37) via the MAPK/ERK signaling pathway (36, 38). Given that iKras"IL-6"/−/− mice had a defect in MAPK/ERK activation, we decided to investigate whether Nrf2 expression was defective in these animals. Indeed, we found robust expression of Nrf2 in PanINs of iKras" mice at all the time points studied, but drastically reduced Nrf2 expression in iKras"IL-6"/−/− lesions at those time points when lesions could still be detected (Fig. 5A). Thus, the presence of oncogenic Kras" was not sufficient to activate the ROS detoxification program, and additional inflammatory signals were required.

To investigate in more detail the effect of IL-6 depletion on the ability of tumor cells to cope with oxidative damage, we used the primary mouse pancreatic cancer cell line, 9805, derived from iKras"p53" mice (24). The in vitro approach allowed us to investigate the effect of IL-6 on the tumor cells, independently from possible indirect effects mediated by components of the stroma. Of note, 9805 cells express the IL-6 receptor (Il6r) in a Kras"-dependent manner, and are thus able to respond to IL-6 signaling (Fig. 5C). The cancer cells were seeded to chamber slides at 50% to 60% confluence and cultured in complete medium containing doxycycline at 1 μg/mL overnight. Then, the cells were subdivided into four groups: control, treated with H2O2 alone to induce intracellular ROS accumulation, treated with H2O2 in the presence of recombinant IL-6, or IL-6 preincubated with an IL-6 antagonist (36). At the 5- to 8-week time point, when most of the changes within the tissue were taking place, iKras" IL-6"/−/− tissues, compared with iKras" had a higher proapoptotic ratio, for Bak/Bcl-x and for Bax/Bcl-x (Supplementary Fig. S5B). In a second set of experiments, we conducted co-immunofluorescent staining with CK19, a ductal and PanIN marker, and amylase, an acinar marker. In iKras" tissues, as expected, we observed almost exclusive expression of CK19 in epithelial cells (Fig. 4A and B). In iKras"IL-6"/−/− tissues, CK19 was prevalent at 3 weeks post-pancreatitis, but by 5 to 8 weeks post-pancreatitis up to one third of the epithelial cells coexpressed CK19 and amylase, and a significant amylase-positive population was observed (Fig. 4A and B). By 17 weeks, CK19-only cells were rare, whereas amylase-positive cells represented the majority of the epithelial population, and some cells coexpressing CK19 and amylase were still present (Fig. 4A and B). The transcription factor Mist1 is a key regulator of acinar cell differentiation (33), and its expression is lost during PanIN formation. Importantly, inactivation of Mist1 in the context of oncogenic Kras accelerates pancreatic carcinogenesis (34), whereas conversely forced expression of Mist1 prevents Kras-driven carcinogenesis (35). As expected, we did not observe any Mist1 expression in the lesions of iKras" mice (Fig. 4C). Similarly, in iKras"IL-6"/−/− lesions at 3 weeks post-pancreatitis, Mist1 expression was absent. However, by 5 weeks post-pancreatitis, Mist1 was expressed in the residual ductal/PanIN structures, thus confirming their mixed differentiation status (Fig. 4C). At later time points, Mist1 expression was confined to acini and excluded from the ducts, as in the normal pancreas.
ERK1/2, even in the absence of exogenous IL-6. However, there was a strong increase of p-ERK1/2 in cells treated with H2O2 in the presence of IL-6, which was reversed by the anti-IL-6 antibody. A similar effect was observed with p-Akt and Nrf2, whereas p-Stat3 did not seem to be affected by the induction of oxidative stress (Fig. 5E). Thus, our data indicate that IL-6 provides protection from oxidative stress.

To determine whether the failure to activate the ROS detoxification program in iKras^+/−/IL-6^−/− mice could explain the lack of PanIN progression, we used a ROS scavenger, N-
acetyl-l-cysteine (NAC). In brief, iKras\(^ {+/\text{IL-6}} \) mice were treated with NAC starting 2 weeks after induction of pancreatitis, and then for 3 weeks (Supplementary Fig. S3D). The mice were harvested at 5 weeks post-pancreatitis. Compared with untreated iKras\(^ {+/\text{IL-6}} \) mice (Fig. 2B), NAC-treated iKras\(^ {+/\text{IL-6}} \) mice revealed partial rescue of carcinogenesis. Their pancreata had extensive PanIN lesions with elevated p-ERK1/2 and p-Stat3 levels, and rescue of proliferation both within the epithelial and the stroma compartments (Supplementary Fig. S5E). In conclusion, the requirement for IL-6 during pancreatic carcinogenesis might be explained, at least in part, by preventing ROS accumulation.

**IL-6 prevents tissue repair following Kras inactivation**

The iKras\(^ + \) mice allow us to turn off Kras\(^ + \) expression at will, thus it is a suitable model to study pancreatic repair following inactivation of the Kras oncogene. To study the role of IL-6 during pancreatic repair, we carried out the following experiment: adult mice were kept on doxycycline for 3 weeks following pancreatitis induction, then some of the animals were harvested, and the others were placed on doxycycline-free chow and water. Of note, 4 to 5 mice per genotype were then harvested 1 day, 3 days, and 2 weeks later (Fig. 6A), based on our previous characterization of the dynamics of pancreas repair in this model (21). In iKras\(^ + \) mice, IL-6 was expressed through most of the repair process, though with decreasing expression levels over time (Supplementary Fig. S6B and S6D). Whole tissue analysis by Western blot indicated that inactivation of oncogenic Kras\(^ + \) led to rapid downregulation of p-ERK1/2 and p-Akt levels within 1 day and to a transient upregulation of cleaved caspase-3 levels, indicating cell death. However, p-Stat3 remained high initially, possibly as a result of its expression in the inflammatory cells that infiltrate the tissue during the repair process (Supplementary Fig. S6C). In fact, immunohistochemistry revealed abundant p-Stat3-positive cells within the stroma compartments (Supplementary Fig. S6E). iKras\(^ {+/\text{IL-6}} \) mice had lower levels of p-ERK1/2, p-Akt, and p-Stat3 than iKras\(^ + \) mice; these pathways were further downregulated upon Kras\(^ + \) inactivation. In contrast, these mice had a higher basal level of cleaved caspase-3, and it further increased in a transient manner following Kras\(^ + \) inactivation. However, cleaved caspase-3 became undetectable as early as 3 days following Kras\(^ + \) inactivation (Supplementary Fig. S6C).

In iKras\(^ + \) mice, the repair process was complete by 2 weeks (Fig. 6B and quantification in 6C). Consistently, PAS positivity was still abundant 1 day after Kras\(^ + \) inactivation, whereas by 3 days, the acinar clusters were mostly lined by PAS-negative inflammatory cells, corresponding to the newly formed acinar cells, enter the cell cycle, as part of the repair process. In iKras\(^ {+/\text{IL-6}} \) pancreata, acinar cells were formed and entered the cell cycle sooner upon Kras inactivation, possibly contributing to the faster recovery process (Supplementary Fig. S8B and S8C; quantification in Supplementary Fig. S8D). Thus, while IL-6 was required for proliferation of PanIN cells and stroma, it was dispensable for—and possibly inhibited—proliferation of normal acinar cells. Our data indicate that IL-6 has a negative effect on pancreatic tissue repair, possibly inhibiting pancreatic acinar differentiation and acinar cell proliferation.

**Therapeutic inhibition of IL-6 causes PanIN regression**

In a final set of experiments, we investigated the effect of blocking IL-6 signaling in KPCY (22, 39) mice using an anti-IL-6-blocking antibody, a strategy that has therapeutic relevance. The KPCY model was chosen because of its proven relevance to clinical response in patients (40), and for its relevance to clinical response in patients (40), and for its
predictable disease progression. Our goal was to treat KPCY mice after PanIN lesions had developed, to determine whether IL-6 was required for PanIN maintenance. Ten-week-old KPCY mice were randomized to two treatment arms, and administered respectively anti-IL-6 antibody or isotype control. The animals were sacrificed 7 days after the beginning of the treatment (see scheme in Fig. 7A). The tissue was then prepared for histopathologic examination. In the control group \((n = 3)\), mice presented with PanINs interspersed within normal exocrine tissue and areas of ADM, as expected on the basis of the disease progression in this model (Fig. 7C). Although the majority of PanINs were of grade 1A-2, PanIN3

**Figure 7.** *In vivo* anti-IL-6 treatment reverses PanIN lesions. A, experimental design; \(n = 3\). B, pathologic analysis. Data represent mean ± SEM; \(n = 3\). C and D, hematoxylin and eosin (H&E) staining of KPCY mice pancreata treated with isotype control (C) or anti-IL-6 antibody (D) 1 week following the beginning of treatment. Scale bar, 100 μm. E, working model indicating the requirement of IL-6 during the maintenance and progression of pancreatic cancer in mice.

**Figure 6.** IL-6 prevents tissue repair following Kras’ inactivation. A, experimental design; \(n = 4–5\). B, hematoxylin and eosin (H&E) staining of iKras/ and iKras/+; IL-6−/− mice pancreata 3 weeks post-pancreatitis and 1 day, 3 days, and 2 weeks following Kras’ inactivation. Scale bar, 50 μm. C, pathologic analysis. Data represent mean ± SEM; \(n = 3–4\). The statistical difference was determined by two-sided Student \(t\) test. *, \(P < 0.05\); **, \(P < 0.01\); †, not significant. D, coimmunofluorescence staining for CK19 (green), amylase (red), SMA (magenta), and 4',6-diamidino-2-phenylindole (DAPI; blue). Scale bar, 25 μm. E, quantification of CK19 and amylase single- or double-positive epithelial cells in iKras/ and iKras/+;IL-6−/− mice pancreata. Data represent mean ± SEM; \(n = 3\). The statistical difference was determined by two-sided Student \(t\) test. *, \(P < 0.05\); **, \(P < 0.01\); †, \(P < 0.001\); ††, \(P < 0.001\). Doxy, doxycycline.
lesions were also observed (Fig. 7B). In contrast, in the anti-IL-6–treated group, the tissue was largely populated by normal acini, with occasional areas of ADM and virtually no PanINs (Fig. 7D and quantification in Fig. 7B). Thus, these results indicate that IL-6 signaling is required for PanIN maintenance once initiated, mimicking the findings obtained in iKras^+/− mice using genetic inactivation of IL-6. Notably, the requirement for IL-6 was maintained even in presence of a loss-of function allele of the tumor suppressor p53.

Discussion
The formation of PanINs, the most common precursor lesions of pancreatic cancer, is accompanied by the accumulation of a desmoplastic stroma with abundant immune infiltrates (28) and by expression of several inflammatory cytokines, including IL-6 (18). It has been suggested that inflammatory environment is an important component of pancreatic cancer (41); however, our mechanistic understanding of the effect inflammation during cancer progression is incomplete. Recent evidence indicates that, at least in mice, the most common cell of origin for pancreatic cancer is an acinar cell that has dedifferentiated to a duct-like cell, often in response to local tissue injury (42, 43). The process of ADM occurs in the pancreas in response to damage such as the induction of acute or chronic pancreatitis. However, the wild-type pancreas is able to undergo tissue repair rapidly and effectively within a short time from the cessation of the injury stimulus. In contrast, pancreata bearing an oncogenic form of Kras are unable to undergo tissue repair, and get "locked" in the ADM status, and progress to PanIN lesions (12). We have previously shown that inactivation of oncogenic Kras at early stages of carcinogenesis allows tissue repair, by allowing redifferentiation of duct-like cells into acinar cells (21). We have also shown that IL-6 is expressed in a Kras-dependent manner in the iKras^+ mouse pancreas. Ras-dependent expression of IL-6 was previously described in multiple cell types (44). Progression toward cancer is accompanied by high levels of IL-6, whereas the repair process correlates with repression of IL-6 expression (21).

Given the important role that IL-6 plays during the initiation of pancreatic cancer (18), we decided to investigate whether it constitutes a key player in modulating the balance between tissue repair and carcinogenesis. Our findings showed that IL-6 was dispensable for pancreatitis-driven PanIN formation, but necessary for the maintenance and progression of the lesions. In fact, in absence of IL-6, the lesions were progressively cleared from the tissue, by a combination of cell-intrinsic mechanisms within the epithelial cells, and extrinsic mechanisms involving epithelial-mesenchymal interactions (see scheme in Fig. 7E).

Within the epithelial cells, we observed extensive apoptotic cell death in absence of IL-6, consistent with previous reports associating IL-6 with cell survival (15), as well as redifferentiation of PanIN cells into normal acini. Moreover, PanIN lesions that formed in the absence of IL-6 failed to activate the Kras effector pathways MAPK and PI3K/Akt and had reduced levels of p-Stat3. The failure to activate the MAPK and PI3K pathways was somewhat surprising, as it could be expected that the presence of constitutively active, oncogenic Kras^+ would bypass any upstream signal. However, our finding is consistent with several recent reports indicating the need for a positive feedback loop to ensure full activation of oncogenic Kras^+ (45–47). Importantly, activation of the MAPK pathway is both sufficient and necessary for pancreatic carcinogenesis in mice (45, 48). An important target of the MAPK signaling pathway in pancreatic cancer is Nrf2 (36), a transcription factor that drives the ROS detoxification program (49). ROS have been associated with carcinogenesis both as a tumor promoter, given the connection between ROS and genome instability (50), and as a barrier to carcinogenesis as ROS accumulation is toxic for the cells (51). Nrf2 deletion in mouse models of pancreatic cancer prevents tumor progression, indicating that the ROS detoxification is an essential step during tumorigenesis (36). Here, we show that, even in the presence of mutant Kras, IL-6 signaling was essential to upregulate Nrf2 expression in PanIN lesions. We then showed, using a primary mouse pancreatic cancer cell line, that failure to upregulate Nrf2 caused oxidative damage and led to tumor cell death. In fact, the failure of iKras^+/− lesions to progress could be rescued, at least partially, by treating the mice with the ROS scavenger NAC. Thus, our results indicate that IL-6 signaling is essential to activate the ROS detoxification program in pancreatic cancer.

In addition to the epithelial changes, important differences also occur within the stroma. Lack of IL-6 affected both the fibroblasts within the stroma, which became unable to maintain their activation status, and the inflammatory microenvironment. In particular, the cytokines IL-1β and granulocyte macrophage colony-stimulating factor (GM-CSF) were expressed at lower levels, and fewer myeloid-derived suppressor cells accumulated within IL-6–deficient tissues. IL-1β can directly drive oncogenesis and suppress cancer immunosurveillance mechanisms via mobilizing myeloid-derived suppressor cells (52). GM-CSF is overexpressed by pancreatic cancer cells and promotes tumor growth by creating a permissive immune environment (31, 32). Thus IL-6 emerges as a key regulator of multiple immune signals that are important for tumor growth.

Our working model is that IL-6 promotes proliferation and survival of neoplastic cells, whereas inhibiting proliferation of acinar cells, as evidenced by our tissue repair experiments. The iKras^+ mouse model has the unique feature of allowing inactivation of oncogenic Kras^+ at will (21). Mice lacking IL-6 expression had accelerated recovery of acinar cells and increased acinar cell proliferation. Thus, IL-6 switches the balance between tissue-repair and carcinogenesis in the pancreas.

Our results have potential therapeutic implications by providing a rationale for the use of IL-6 inhibitors in pancreatic cancer, at a time when several inhibitors are reaching the clinic (for review see ref. 53). In fact, our proof-of-principle test in KPCY mice showed that IL-6 blocked PanIN progression in this model. Expression patterns of IL-6 in human tissues and blood should be explored in a large panel of human cancers, as the current published findings relied on a relatively small sample set (14), and compared with the conclusions made in mouse models. Further work will also be needed to
IL-6 Is Required for Pancreatic Cancer Progression

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References


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Authors' Contributions
Conception and design: Y. Zhang, B.R. Zetter, B.Z. Stanger, I. Chung, A.D. Rhim, M.P. di Magliano
Development of methodology: Y. Zhang, M.P. di Magliano
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Zhang, M.A. Collins, F. Bednar, S. Rakshit, B.R. Zetter, A.D. Rhim, M.P. di Magliano
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Zhang, W. Yan, F. Bednar, B.R. Zetter, A.D. Rhim, M.P. di Magliano
Writing, review, and/or revision of the manuscript: Y. Zhang, M.A. Collins, F. Bednar, B.R. Zetter, B.Z. Stanger, I. Chung, A.D. Rhim, M.P. di Magliano
Study supervision: B.R. Zetter, B.Z. Stanger, M.P. di Magliano

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Interleukin-6 Is Required for Pancreatic Cancer Progression by Promoting MAPK Signaling Activation and Oxidative Stress Resistance

Yaqing Zhang, Wei Yan, Meredith A. Collins, et al.


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