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Article

Oncogenic Kras Activates a Hematopoietic-to-Epithelial IL-17 Signaling Axis in Preinvasive Pancreatic Neoplasia

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SUMMARY

Many human cancers are dramatically accelerated by chronic inflammation. However, the specific cellular and molecular elements mediating this effect remain largely unknown. Using a murine model of pancreatic intraepithelial neoplasia (PanIN), we found that $Kras^{G12D}$ induces expression of functional IL-17 receptors on PanIN epithelial cells and also stimulates infiltration of the pancreatic stroma by IL-17-producing immune cells. Both effects are augmented by associated chronic pancreatitis, resulting in functional in vivo changes in PanIN epithelial gene expression. Forced IL-17 overexpression dramatically accelerates PanIN initiation and progression, while inhibition of IL-17 signaling using genetic or pharmacologic techniques effectively prevents PanIN formation. Together, these studies suggest that a hematopoietic-to-epithelial IL-17 signaling axis is a potent and requisite driver of PanIN formation.

INTRODUCTION

A hallmark of many solid tumors is the prominent fibrocellular stroma surrounding the neoplastic epithelium. This stromal expansion is especially dramatic in both invasive pancreatic cancer and its noninvasive precursor lesions. Recent studies suggest that this stroma may be required for tumor maintenance, progression, and resistance to chemotherapy (Jacobetz et al., 2013; Olive et al., 2009; Provenzano et al., 2012). However, information has only recently begun to emerge regarding the cellular and molecular elements mediating this stromal effect. Much recent attention has been focused on the role of activated stromal fibroblasts in pancreatic tumorigenesis, as these cells produce a host of factors capable of influencing adjacent malignant epithelium, including TGF-β and Notch pathway ligands (Bailey and Leach, 2012; Chu et al., 2007). In addition to activated fibroblasts, analysis of immune cell infiltration has revealed a prominent inflammatory infiltrate comprised mainly of immunosuppressive myeloid cells, recruited in response to even the lowest grade, preinvasive pancreatic lesions (Clark et al., 2007).

While oncogenic Kras itself can induce spontaneous infiltration of immune cells, the additional presence of antecedent and/or concomitant chronic inflammation also appears to dramatically modify the initiation and progression of pancreatic

Significance

Pancreatic cancer remains one of the most deadly human malignancies. In addition to the malignant epithelium, pancreatic tumors are characterized by dramatic stromal expansion involving mesenchymal and inflammatory cell types. While recent studies suggest that this stromal reaction is required for tumor maintenance and progression, the specific molecular and cellular components responsible for this effect are largely unknown. Here, we show that IL-17 production from $CD4^+$ T cells and γδT cells is required for the initiation and progression of early pancreatic cancer. Inhibition of IL-17 signaling effectively prevents the initiation of pancreatic neoplasia, suggesting that therapeutic targeting of this hematopoietic-to-epithelial IL-17 signaling axis may represent a potentially effective strategy for the chemoprevention and/or treatment of early pancreatic cancer.

cancer. In mice, pancreatic cancer progression is accelerated in the presence of associated chronic pancreatitis (Guerra et al., 2007; Habbe et al., 2008; Kopp et al., 2012), and chronic inflammation itself is capable of inducing metaplastic changes resembling pancreatic intraepithelial neoplasia (PanIN) (Strobel et al., 2007). In humans, many of the known risks factors for pancreatic cancer, including chronic pancreatitis, diabetes, alcohol consumption, cystic fibrosis, and tobacco use, are all commonly characterized by the induction of chronic inflammation (Greer and Whitcomb, 2009; Hassan et al., 2007; Maisonneuve et al., 2007; Wittel et al., 2006).

Among the inflammatory cell types potentially mediating this effect, a subset of IL-17-producing T helper cells (T_H17) has been shown to play an active role in both chronic inflammation (Kimura et al., 2007) and inflammation induced-tumorigenesis (Wu et al., 2009; Xiao et al., 2009). T_H17 cell differentiation requires IL-6 and TGF-β, and both factors are abundant in the pancreatic tumor microenvironment (Lesina et al., 2011; Löhr et al., 2001). Based on the strong association between chronic inflammation and pancreatic cancer, we therefore sought to identify the role of IL-17 producing hematopoietic cells in the earliest stages of pancreatic neoplasia.

RESULTS

Human Pancreatic Cancer Precursor Lesions Are Infiltrated by IL-17-Producing T Cells and Overexpress IL-17 Receptor A

To better characterize inflammatory cell types participating in early pancreatic neoplasia, we labeled human tissue arrays using antibodies against RORγt, a transcription factor that directs differentiation of IL-17-producing T cells (Ivanov et al., 2006). While RORγt$^+$ cells were rarely identified in normal human pancreas, they were abundant in human pancreatic preneoplastic tissue. RORγt$^+$ cells were localized in the stroma immediately adjacent to areas of acinar-ductal metaplasia (ADM), as well as early or advanced PanIN (Figure 1A). Compared to normal, we calculated an ~25-fold increase in the abundance of RORγt$^+$ cells in human chronic pancreatitis and an ~50-fold increase associated with PanIN lesions (Figure S1 available online).

In order to identify relevant cell types capable of responding to IL-17, we examined the expression of the IL-17 Receptor A (IL-17RA) in the same human tissue arrays and we detected no IL-17RA expression in normal acinar tissue, low levels of IL-17RA expression in ADMs and higher levels in PanINs (Figures 1B–1E). In transitional lesions containing both cuboidal ADM cells and tall, columnar PanIN cells, we observed high level staining in the PanIN component, but low staining in the adjacent cuboidal ADM cells (Figures 1D and 1F).

Oncogenic Kras and Chronic Pancreatitis Synergistically Recruit T_H17 and IL-17$^+$/γδT Cells to the Pancreatic Microenvironment

To functionally determine the significance of IL-17 signaling in early pancreatic neoplasia, we utilized *Mist1*$^{CreERT2/+}$; LSL-*Kras*^{G12D} (KC^{iMist1}) or control *Mist1*$^{CreERT2/+}$ (C^{iMist1}) mice treated with and without cerulein (Habbe et al., 2008) (Figure 2A). In the setting of concomitant cerulein-induced chronic pancreatitis, these mice rapidly develop murine PanIN (mPanIN) within 4 weeks following Kras activation, with progression to advanced mPanIN by 9 weeks posttamoxifen (Figures S2A–S2D). Similar to other transgenic systems used for the induction of pancreatic tumorigenesis (Corcoran et al., 2011), we detected phosphorylation of Stat3 in tamoxifen-treated KC^{iMist1} mice, both within the mPanIN epithelium and in the surrounding stroma (Figure S2E). When we assayed media conditioned by whole pancreatic suspensions, we observed a Kras^{G12D}- and chronic pancreatitis-dependent increase in the production of IL-6, consistent with prior reports (Corcoran et al., 2011), and IL-17A (Figures S2F and S2G). Based on the known roles for IL-6 and Stat3 in the peripheral differentiation of naive CD4$^+$ T helper cells into T_H17 cells, we performed flow cytometry on single cells obtained from collagenase-digested pancreas and observed that the relative and absolute numbers of CD45$^+$ hematopoietic cells displaying intracellular staining for IL-17A were increased in the mice with chronic pancreatitis (C^{iMist1} + CP) or oncogenic Kras (KC^{iMist1}). A synergistic increase in the number of IL-17A expressing cells was observed in mice in which Kras^{G12D} activation was combined with chronic pancreatitis (KC^{iMist1} + CP) (Figure 2B). We further assessed multiple cellular markers to more definitively characterize IL-17A-expressing cells and found that CD4$^+$ T cells and γδT cells were the two types of cells that also stained for intracellular IL-17A (Figure 2C). We observed no colabeling of IL-17 with Gr1, CD11b, CD117, or NKp46 (data not shown), ruling out macrophages, neutrophils, NK cells, mast cells, and MDSCs as significant sources of IL-17.

In attempting to quantify the relative expression of IL-17 by T_H17 and γδT cells, we noted that while CD4$^+$ T cells were approximately five times more abundant than γδT cells within the pancreatic microenvironment from KC^{iMist1} + CP mice, only ~10% of CD4$^+$ T cells expressed IL-17A, compared to ~50% of γδT cells (Figures 2C and S2H), suggesting that the contribution of IL-17A from both cellular sources may be similar. In order to determine how the expression of IL-17A and other cytokines was dynamically regulated within the CD4$^+$ and γδT cell compartments during mPanIN formation, we performed quantitative RT-PCR (qRT-PCR) for IL-17A, IL-22, IFNγ, IL-4, and TNFα on RNA isolated from fluorescence-activated cell sorting (FACS)-sorted CD4$^+$ and γδTCR$^+$ cells harvested from mice with either chronic pancreatitis alone (C^{iMist1} + CP), oncogenic Kras activation alone (KC^{iMist1}) or oncogenic Kras activation combined with chronic pancreatitis (KC^{iMist1} + CP). This analysis revealed that the combination of oncogenic Kras and chronic pancreatitis synergistically and dramatically activated expression of IL-17A and IL-22 in both the CD4$^+$ and γδTCR$^+$ populations, with less pronounced effects observed on expression of IFNγ, IL-4, and TNFα (Figure 2D).

To gain insight into a possible functional contribution of IL-17-producing cells during early pancreatic neoplasia, we depleted CD4$^+$ T cells from KC^{iMist1} + CP mice with weekly GK1.5 injections. This resulted in a significant delay in PanIN formation, with no overt change in the overall magnitude of the associated stromal response (Figures 2E–2G). These findings led us to hypothesize that IL-17-producing T cells might exert a proneoplastic influence during PanIN formation.

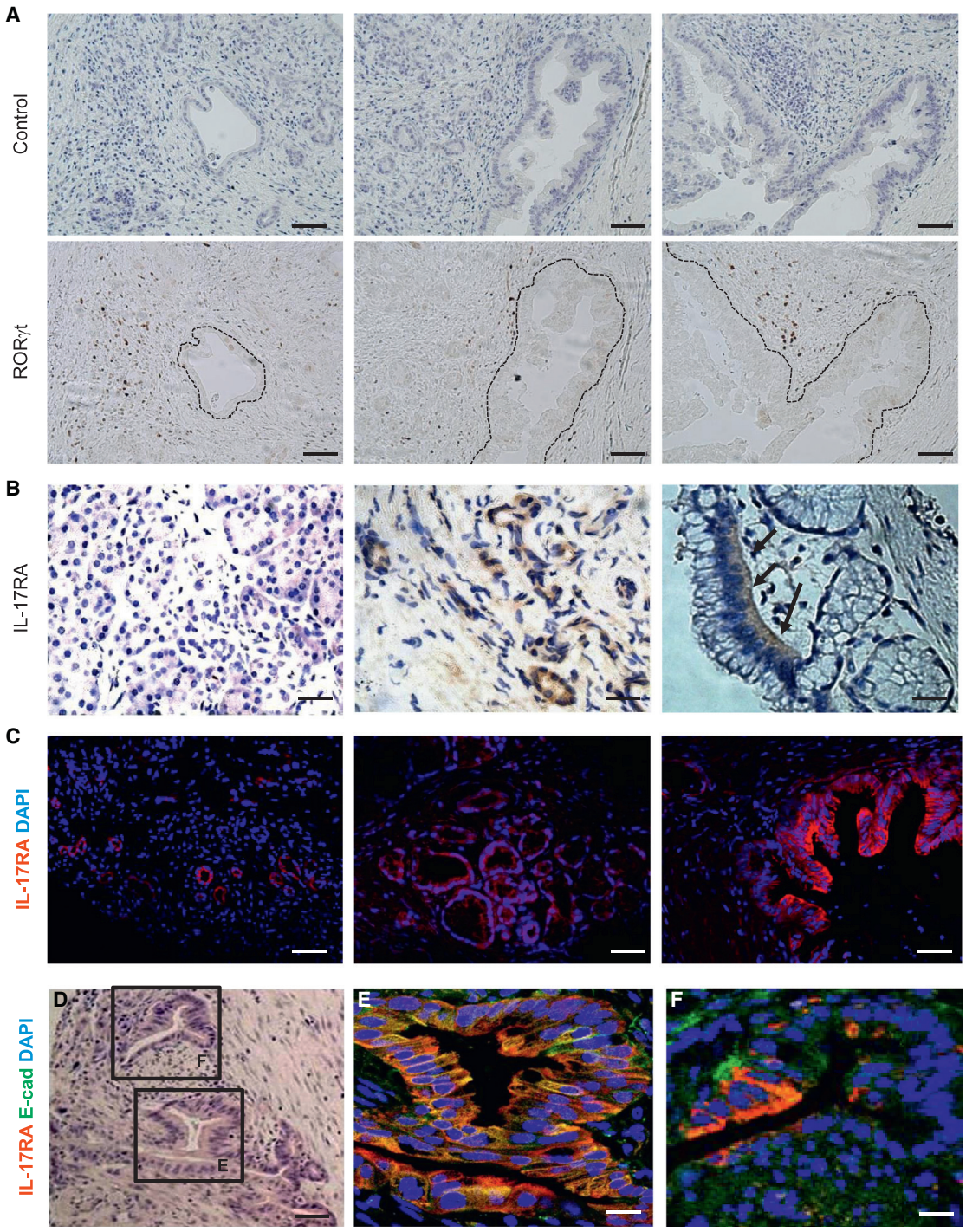

Figure 1. Human Pancreatic Cancer Precursor Lesions Are Infiltrated by IL-17-Producing T Cells and Overexpress the IL-17 Receptor A

(A) Representative sections of RORγt$^+$ cells infiltrating human ADM (left), early PanIN (middle), and advanced PanIN (right). Control sections treated with hematoxylin for morphological reference are shown on the top panels. Scale bars represent 50 μm.

(B) Immunohistochemical detection of IL-17RA in normal acinar tissue (left, scale bar represents 50 μm), ADMs (middle, scale bar represents 100 μm), and PanIN lesion (right, scale bar represents 150 μm).

(C) Immunofluorescent detection of IL-17RA on human ADMs (left), early PanIN (middle), and advanced PanIN (right). Scale bars represent 50 μm.

(D) Hematoxylin and eosin (H&E) staining of human tissue containing transitional elements comprised of both ADM and PanIN. Scale bars represent 50 μm.

(E and F) Immunofluorescent detection of IL-17RA on a PanIN (E) and on a transitional ADM (F). Scale bars represent 150 μm.

See also Figure S1.

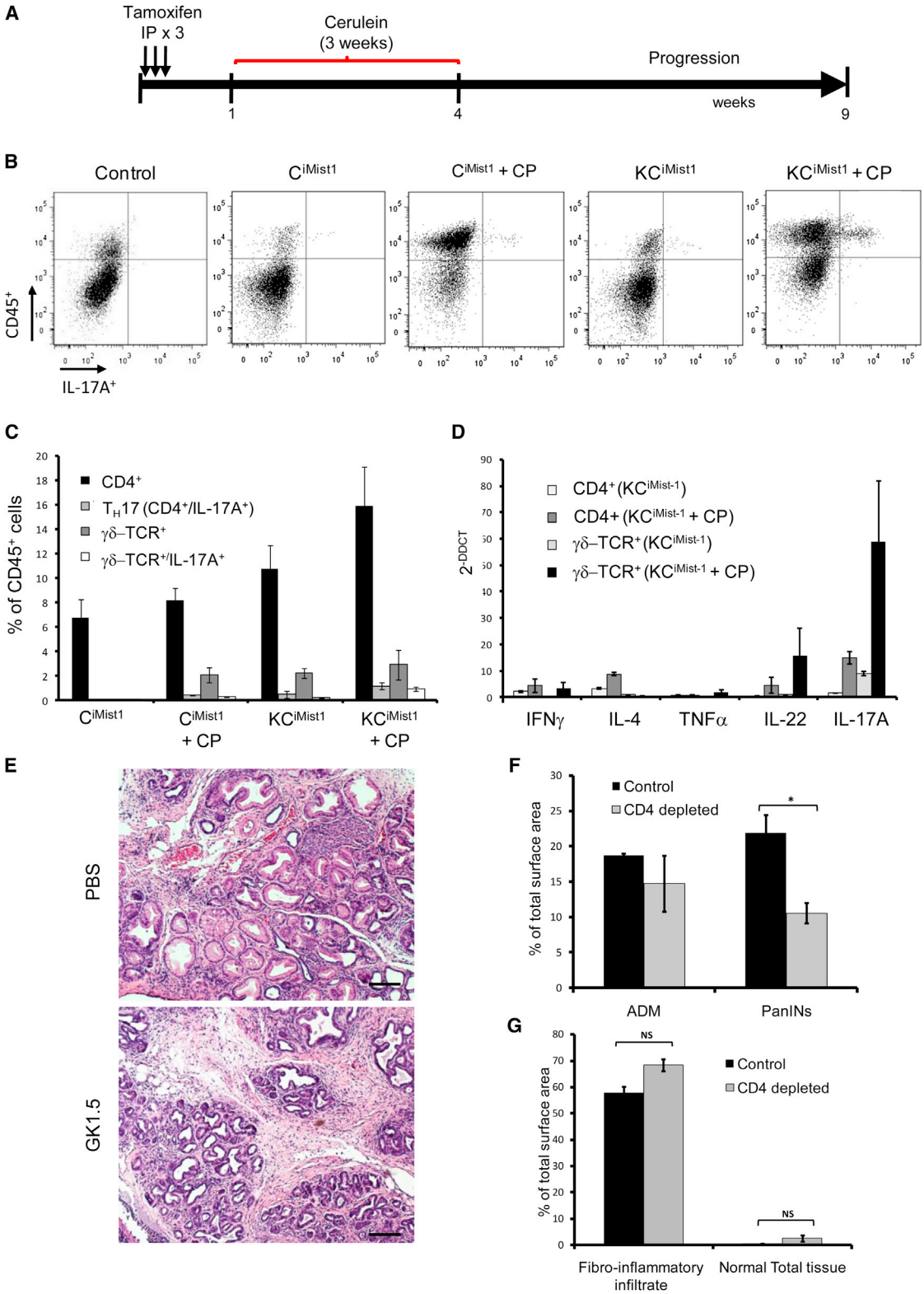

Figure 2. Oncogenic Kras and Chronic Pancreatitis Synergistically Recruit $T_H17$and IL-17$^+$ γδT Cells to the PanIN Microenvironment

(A) Protocol followed for tamoxifen-mediated $Kras^{G12D}$ activation and cerulein-mediated induction of chronic pancreatitis in KC^{iMist1} and C^{iMist1} mice.

(B) Flow cytometry dot plots for dual labeling of CD45 and intracellular IL-17A on dispersed pancreatic cells from the indicated mice. Control flow chart (Left) represents pancreatic cells from a $KC^{iMist-1}$ mouse stained for CD45 only.

(legend continued on next page)

Pancreatic Overexpression of IL-17A Results in Accelerated PanIN Initiation and Progression

In order to gain more insights into possible influences of IL-17A on PanIN initiation and progression, we next performed gain-of-function studies by injecting IL-17A encoding (AdIL-17A) or control (Ad-EGFP or Ad-Luc) adenoviruses into the pancreas in a manner that resulted in relatively uniform adenoviral gene delivery (Figures 3A and S3A). Following injection of AdIL-17, we observed a 40-fold increase in IL-17A in media conditioned by dissociated pancreatic cells (Figure 3B). To determine the effect of IL-17A overexpression on PanIN formation, KC^{iMist1} mice underwent pancreatic adenoviral infection one week following tamoxifen induction of $Kras^{G12D}$ expression (Figure 3A). These studies were performed in the absence of associated cerulein pancreatitis. Six weeks later (7 weeks posttamoxifen), mice were sacrificed and the pancreas was processed for histopathologic examination and morphometric quantification of total surface area occupied by ADM, early PanIN, late PanIN, and fibroinflammatory stroma. In the absence of associated cerulein pancreatitis, control pancreas displayed only focal changes comprised predominantly of ADM, with rare associated early PanIN and minimal stromal expansion (Figures 3C and 3D). In contrast, overexpression of IL-17 resulted in a dramatic increase of ADM and PanIN formation, as well as markedly enhanced stromal expansion as assessed by Masson's trichrome staining (Figures 3E and 3F). Mice infected with AdIL-17A displayed a >4-fold increase in ADM compared to mice infected with Ad-Luc (3.97 ± 0.89 versus 0.95% ± 0.24%) and a >100-fold increase in early PanINs (7.5 ± 2.48 versus 0.07% ± 0.14%) (Figure 3G). Advanced PanINs were absent in the Ad-Luc-infected control pancreas but occupied 0.75% ± 0.29% of total surface area in pancreas infected with AdIL-17A (Figure 3G). Using the same quantification method, the pancreatic surface area occupied by fibroinflammatory stroma was found to be 20-fold greater in mice infected with AdIL-17 compared to mice infected with Ad-Luc (31.61 ± 8.31 versus 1.56% ± 0.56%). This IL-17A-accelerated phenotype, in the absence of cerulein-induced chronic pancreatitis, was accompanied by an associated reduction in surface area displaying normal histology (56.15% ± 10.62% for AdIL-17A versus 97.4% ± 0.71% for Ad-Luc) (Figure 3H). To rule out an inflammatory effect induced by adenovirus, we further confirmed no differences between Ad-Luc- and PBS-injected animals in terms of pancreatic area occupied by either inflammation or preneoplastic lesions (Figures S3B and S3C). Finally, by crossing KC^{iMist1} mice with a $Rosa26^{mTmG}$ lineage tracing mouse line to produce $KC^{iMist1}G$ mice, we were able to confirm that IL-17 accelerated PanINs were derived from the $Mist1^+$ acinar compartment (Figure S3D). In addition to accelerating the appearance and progression of epithelial PanINs, IL-17A overexpression also accelerated the appearance of GFP^+, E-cadherin$^-$ cells entering the stroma through a process of early EMT (see arrows in Figure S3D) (Rhim et al., 2012). These results demonstrate that IL-17A is capable of dramatically accelerating PanIN initiation and/or progression, in a manner similar to that observed for cerulein pancreatitis.

Genetic Ablation of IL-17A within the Hematopoietic Compartment Results in Delayed PanIN Initiation and Progression

The above data suggest that T_H17 and IL-$17^+/\gamma\delta$T cells recruited to PanIN-forming pancreas may play a functionally significant role in driving PanIN progression. In order to directly test this hypothesis, we eliminated hematopoietic IL-17A production in KC^{iMist1} mice by lethal irradiation followed by rescue with transplanted bone marrow (BM) harvested from IL-17A knockout mice (KC^{iMist1}/IL-17^{KO} BM). To control for treatment effects unrelated to IL-17, we similarly transplanted additional irradiated KC^{iMist1} mice with bone marrow harvested from wild-type mice (KC^{iMist1}/IL-17^{WT} BM). Mice undergoing lethal irradiation and bone marrow transplant displayed an element of radiation-induced pancreatic inflammation (Figure S4), obviating the need for the induction of additional inflammation using cerulein. Transplants were performed on mice at 8–10 weeks of age, and tamoxifen induction of $Kras^{G12D}$ expression was performed 8 weeks following bone marrow transplantation (Figure 4A). Immediately prior to proceeding with tamoxifen injections, we confirmed functional bone marrow chimerism in KC^{iMist1}/IL-17^{KO} mice by sacrificing a cohort of these mice, isolating their splenocytes, placing them in T_H17 polarization conditions and restimulating them with phorbol 12-myristate 13-acetate (PMA)/Ionomycin. Using flow cytometry for $CD45^+$ and intracellular IL-17A, we confirmed that KC^{iMist1}/IL-17^{KO} BM had indeed been reconstituted with IL-$17A^{KO}$ bone marrow, as their splenocytes failed to produce significant IL-17A after 1 week of T_H17 polarization and restimulation (Figure 4B). Eight weeks after Kras activation (or 16 weeks after the transplantation), mice were sacrificed and pancreatic tissue was subjected to morphometric analysis to quantify the total pancreatic surface area occupied by ADM, PanIN, and fibroinflammatory stroma. KC^{iMist1}/IL-17^{KO} BM mice displayed a 4-fold reduction in pancreatic surface occupied by ADMs (2.41 ± 0.7 versus 11.25% ± 3.19%) and a near complete prevention of PanIN formation (0.07 ± 0.07 versus 4.62% ± 0.76%) (Figures 4C–4F and 4G). For PanIN identification, Alcian blue was used to confirm the presence of mucin in the apical cytoplasm of PanIN cells (Figures 4H and 4I). Masson's trichrome staining demonstrated a decrease in collagen deposition in the pancreas of KC^{iMist1}/IL-17^{KO} BM mice (Figures 4J and 4K) and quantification of the pancreatic fibroinflammatory stromal component showed that KC^{iMist1}/IL-17^{KO} BM mice displayed less prominent stromal

(C) Quantification of $CD4^+$ T cells, $\gamma\delta$T cells and their double staining with intracellular IL-17A from the indicated mice. T_H17 cells were defined as $CD45^+$/IL-$17A^+$ cells. Results are shown as percent of $CD45^+$ cells ± SEM (n = 3–4).
(D) RT-PCR-based quantification of IFNγ, IL-4, TNFα, IL-22, and IL-17A in $CD4^+$ and $\gamma\delta$T cells sorted by FACS from the pancreas of KC^{iMist1} and KC^{iMist1}+ CP mice. Data was normalized to C^{iMist1} + CP mice and results were presented as relative expression of cytokines/ sorted cell. SEM from triplicates is shown.
(E) Representative H&E staining of pancreatic tissue sections from KC^{iMist1}+ CP mice that received PBS or GK1.5 injections at 7 weeks after Kras activation. Scale bars represent 70 μm.
(F and G) Tridimensional quantification of fractional cross sectional area occupied by ADMs or PanINs (F) or occupied by fibroinflammatory stroma or normal tissue (G) in KC^{iMist1} that received PBS or GK1.5 injection. Results are shown as mean ± SEM (n = 4) (*$p < 0.05$; NS, no statistical significance).
See also Figure S2.

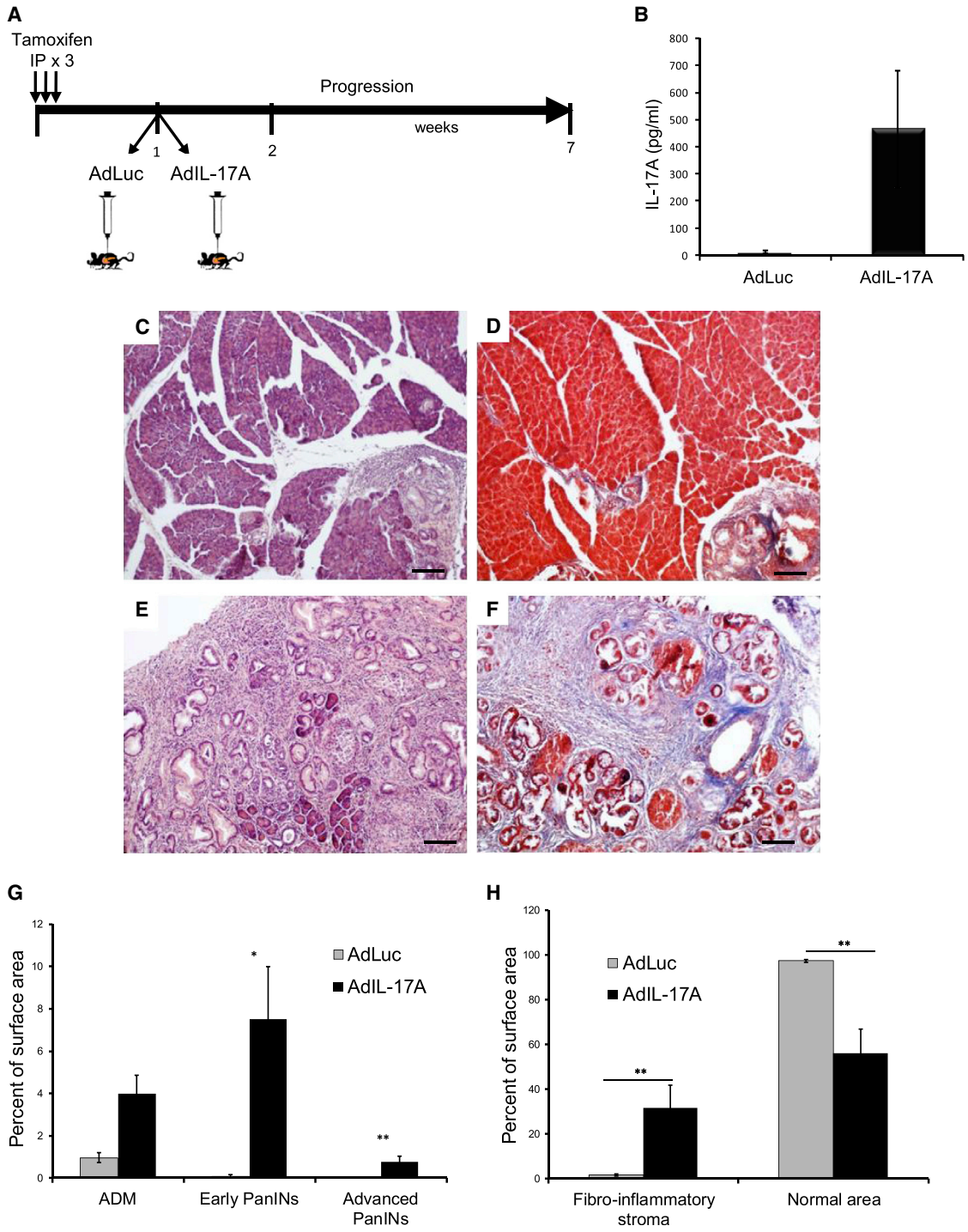

Figure 3. Pancreatic Overexpression of IL-17A Results in Increased Tumor Initiation and Progression

(A) Protocol for Kras activation and delivery of adenovirus to KC^{iMist1} mouse pancreas.

(B) ELISA-based quantification of IL-17A production by dispersed pancreatic cells isolated 1 week following injection of the indicated adenovirus. Results are shown as mean concentration ± SEM (n = 4).

(C–F) Representative H&E (C and E) or Masson's trichrome (D and F) staining of pancreatic tissue harvested from KC^{iMist1} mice injected with Ad-Luc (C and D) or AdIL-17A (E and F) at 7 weeks after Kras activation. Scale bars represent 70 μm.

(G and H) Quantification of fractional cross sectional area occupied by ADM, early PanIN, or advanced PanIN (G) or occupied by fibroinflammatory stroma or normal tissue (H) in KC^{iMist1} mice infected with Ad-Luc versus AdIL-17A. Results are shown as mean ± SEM (n = 9–10) (*$p < 0.05$; **$p < 0.01$).

See also Figure S3.

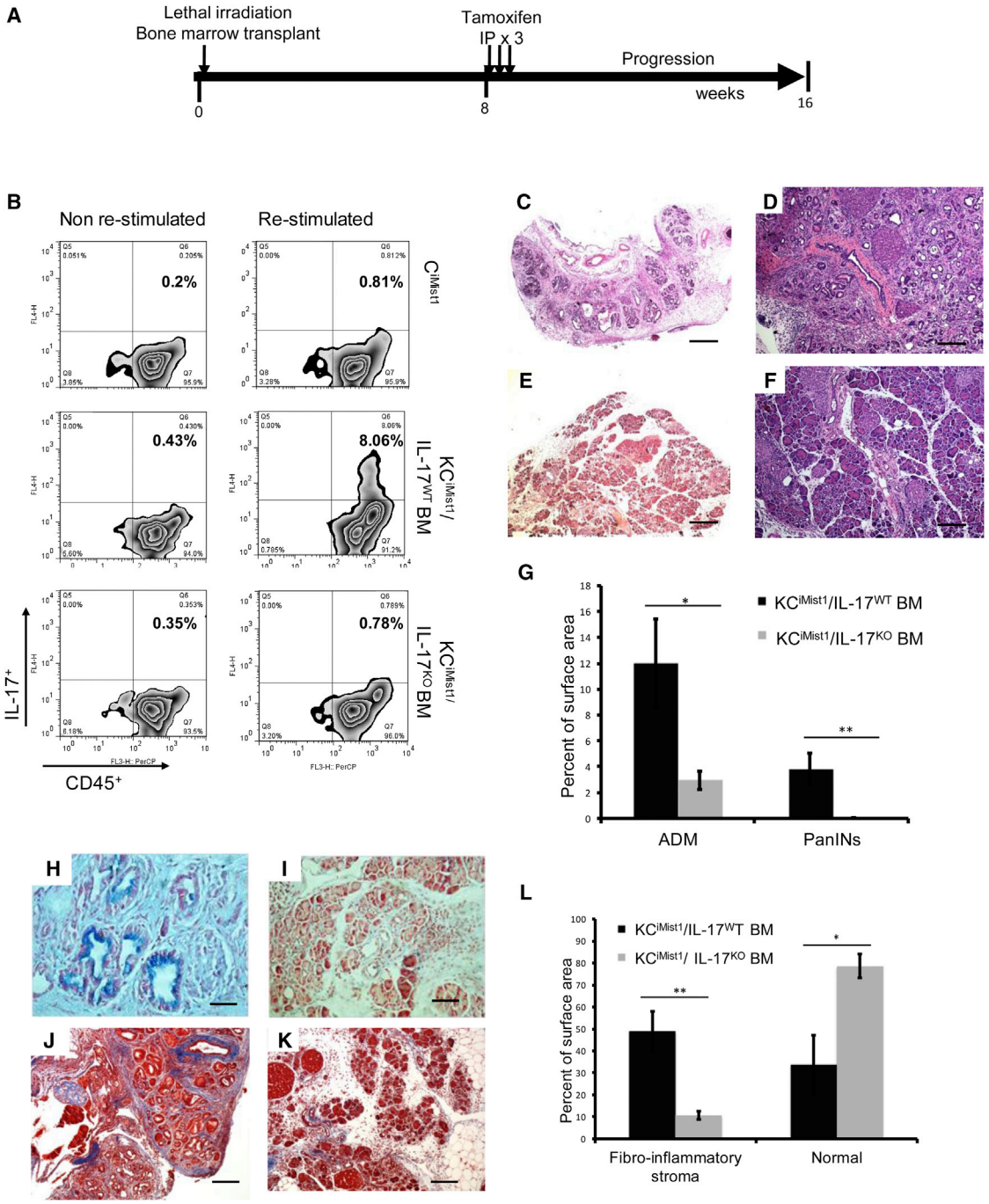

Figure 4. Genetic Ablation of IL-17A within the Hematopoietic Compartment Results in Delayed PanIN Initiation and Progression

(A) Protocol for the generation of $KC^{iMist\text{-}1}$/IL-$17A^{WT}$ BM and $KC^{iMist\text{-}1}$/IL-$17A^{KO}$ BM chimeric animals.

(B) Eight weeks following lethal irradiation and BM transplantation as indicated, splenocytes were cultured in T_H17 polarization conditions and restimulated with PMA/Ionomycin. Bone marrow chimerism was assessed by flow cytometry on polarized splenocytes labeled for cell surface CD45 and intracellular IL-17A. Double-positive cells were quantified and results expressed as percent of total splenocytes.

(C–F) Representative H&E staining on pancreatic tissue sections from $KC^{iMist\text{-}1}$/IL-$17A^{WT}$ BM (C and D) and $KC^{iMist\text{-}1}$/IL-$17A^{KO}$ BM (E and F) mice at 8 weeks following $Kras^{G12D}$ activation. Scale bars represent 400 μm (C and E) and 80 μm (D and E).

(G) Quantification of fractional cross-sectional area occupied by ADM or PanIN in the pancreas of indicated mice. Results are shown as mean ± SEM (n = 5–7) (*$p < 0.05$; **$p < 0.01$).

(H and I) Alcian blue staining for mucins on pancreatic tissue sections from $KC^{iMist\text{-}1}$/IL-$17A^{WT}$ BM (H) and $KC^{iMist\text{-}1}$/IL-$17A^{KO}$ BM (I) mice. Scale bars represent 80 μm.

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expansion than that observed in KC^{iMist1}/IL-17^{WT} BM mice (12.69 ± 3.04 versus 56.22% ± 6.05%) (Figure 4L). Correspondingly, areas from KC^{iMist1}/IL-17^{KO} BM mice displayed a significantly larger pancreatic surface area characterized by normal histology (84.07% ± 3.6% for KC^{iMist1}/IL-17^{KO} BM versus 27.08% ± 9.8% for KC^{iMist1}/IL-17^{WT} BM) (Figure 4L). These data confirm the functional significance of IL-17 production by T_H17 and IL-$17^+/\gamma\delta$T cells in the pathogenesis of early pancreatic neoplasia.

Pharmacological Neutralization of IL-17 Pathway Results in Delayed Initiation and Progression of PanINs

Antibody-based neutralization of IL-17 signaling is currently being evaluated in the treatment of human autoimmune disease (Genovese et al., 2010; Leonardi et al., 2012; Papp et al., 2012; van den Berg and Miossec, 2009). In order to determine whether this clinically-relevant mode of IL-17 inhibition might similarly abrogate PanIN initiation and progression, we treated KC^{iMist1} + CP mice with a cocktail of monoclonal antibodies directed against IL-17RA (Stagg et al., 2011; Teng et al., 2012) and the cytokines IL-17A (Sarkar et al., 2009) and IL-17F. Antibodies were administered by weekly intraperitoneal (IP) injection, with treatment starting 48 hr prior to tamoxifen induction and followed by 3 weeks of cerulein injections to induce chronic pancreatitis (Figure 5A). Morphometric analysis revealed that the fraction of total pancreatic surface area occupied by ADMs was identical in control mice and in mice receiving neutralizing antibodies. However, there was a 4-fold decrease in surface area occupied by early PanINs (6.5 ± 3.69 versus 26.41% ± 4.67%) and a >3-fold reduction in advanced PanINs (1.17 ± 1.1 versus 4.86 ± 0.91) in KC^{iMist1} + CP mice treated with IL-17 pathway neutralizing antibodies compared to control mice (Figures 5B and 5C). There was a modest decrease in fibroinflammatory stromal area in antibody-treated mice (19.3 ± 8.1 versus 32.4 ± 1.3) but this did not reach statistical significance (Figure 5D). Similarly, the fraction of total pancreatic surface area displaying normal histology was doubled in mice treated with neutralizing antibodies compared to controls (64.67 ± 14.4 versus 32.1% ± 6.03%) (Figure 5D). When we performed flow cytometry analysis for multiple cellular types, we detected a decrease in pancreatic infiltration by MDSC, neutrophils and macrophages in mice that received IL-17 neutralizing antibodies (Figure S5). As with genetic ablation of hematopoietic IL-17, these studies confirm a critical role for endogenous IL-17 signaling in PanIN initiation and progression and further suggest that currently available, clinical-grade neutralizing antibodies may represent a viable option for pancreatic cancer prevention and/or treatment.

IL-17RA Expression Is Induced in Murine Pancreatic Epithelium by Oncogenic Kras Activation

We next sought to determine whether the influence of IL-17 on PanIN initiation and progression was mediated directly on PanIN epithelial cells. Using a well characterized anti-IL-17RA antibody (Figures S6A–S6C), we detected minimal pancreatic epithelial expression of IL-17RA by immunofluorescent labeling of pancreatic tissue harvested from either C^{iMist1} or C^{iMist1}+CP mice (Figures 6A and 6B). In contrast, we observed significant IL-17RA expression on the basal membrane of ADM and mPanIN epithelial cells in KC^{iMist1} mice (Figure 6C) and even more intense labeling of the more abundant ADMs and mPanINs observed in KC^{iMist1} + CP mice (Figure 6D). Furthermore, selected pancreatic cells undergoing EMT were found to express IL-17RA, as assessed in $KC^{iMist-1}G$ + CP mouse pancreas by colabeling for IL-17RA and GFP (Figure S6D). Using E-cadherin labeling to discern individual cells in KC^{iMist1} mouse pancreas, we found that 9.3% ± 4.09% of ADM cells and 23.5% ± 8.07% of PanIN cells stained positive for IL-17RA (Figures 6E and S6E, left panel). In KC^{iMist1} + CP mice, these fractions increased to 33.4% ± 4.52% for ADM cells and 50.5% ± 9.03% PanIN cells (Figures 6E and S6E, right panel). To further confirm and validate the expression of IL-17RA in the oncogenic epithelium, we examined IL-17RA expression by qRT-PCR and flow cytometry. In order to eliminate normal ductal epithelial cells from this analysis, we again utilized $Rosa26^{mTmG}$ mice, generating $KC^{iMist1}G$ mice in which GFP effectively marked cells undergoing effective Cre-based recombination (Figure S6F). Three months following tamoxifen administration, we observed a dramatic increase in IL-17RA expression in FACS-isolated PanIN epithelial cells (Figure 6F). Flow cytometry on single cells harvested from $KC^{iMist1}G$ and control $C^{iMist1}G$ mice also revealed a 250-fold increase (2.48% versus 0.01%) in the number of GFP^+ cells expressing IL-17RA in $KC^{iMist1}G$ mouse pancreas compared to $C^{iMist1}G$ controls, indicating cell-autonomous activation of IL-17RA by oncogenic Kras (Figure 6G). The induction of IL-17RA expression by oncogenic Kras was also observed in the context of chronic pancreatitis, with a >60-fold increase in the fraction of GFP^+/IL-$17RA^+$ cells observed in $KC^{iMist1}G$ + CP mouse pancreas at 2 months following Kras activation and a >100-fold increase at 4 months (Figure 6H).

$Kras^{G12D}$-Induced Activation of IL-17RA Expression on PanIN Epithelial Cells Is Associated with In Vivo Functional Responses to IL-17

In order to assess the functionality of IL-17 receptors on oncogenic pancreatic epithelium, we treated $KC^{iMist1}G$+ CP mice that had already developed mPanIN lesions with a cocktail of monoclonal antibodies directed against IL-17RA and the cytokines IL-17A and IL-17F. Beginning at 6 weeks following Kras activation, antibodies were administered by two IP injections during the week prior to sacrifice (Figure 7A). At week 7, mice were sacrificed and GFP^+ mPanIN epithelial cells were isolated by FACS. Microarray-based whole transcriptome expression analysis was performed comparing GFP^+ cells sorted from mice that had received IL-17 neutralizing antibodies versus mice that received IgG isotype control antibodies. When differentially expressed genes were subjected to Ingenuity pathway

(J and K) Masson's trichrome staining for collagen deposition in pancreas from $KC^{iMist-1}$/IL-$17A^{WT}$ BM (J) and $KC^{iMist-1}$/IL-$17A^{KO}$ BM (K) mice. Scale bars represent 40 μm.

(L) Quantification of fractional cross-sectional area occupied by fibroinflammatory stroma or normal tissue in the pancreas of indicated mice. Results are shown as mean ± SEM (n = 5–7) (*$p < 0.05$; **$p < 0.01$).

See also Figure S4.

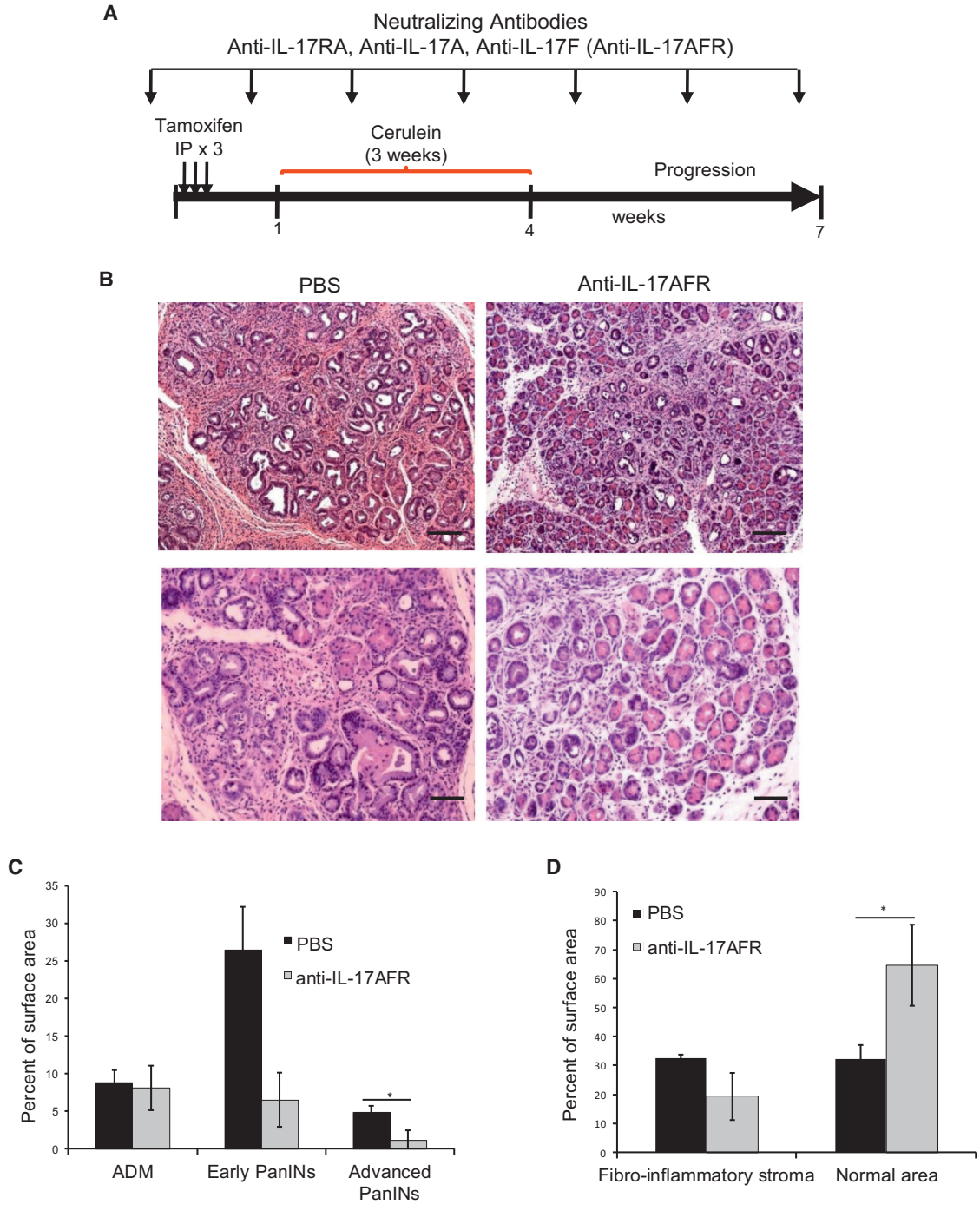

Figure 5. Pharmacological Neutralization of IL-17 Pathway Results in Delayed Initiation and Progression of PanINs

(A) Protocol followed for induction of $Kras^{G12D}$ expression and administration of IL-17 pathway neutralizing antibodies to KC^{iMist1} mice.

(B) Representative H&E staining on pancreatic tissue sections from KC^{iMist1} mice that received IL-17RA, IL-17A, and IL-17F neutralizing antibodies or PBS injection at 7 weeks after Kras activation. Scale bars represent 80 μm for top panels, 40 μm for bottom panels.

(C and D) Quantification of fractional cross sectional area occupied by ADMs or PanINs (C) or occupied by fibroinflammatory stroma or normal tissue (D) in KC^{iMist1} that received PBS injections (black bars) versus KC^{iMist1} that received the combination of IL-17RA, IL-17A, and IL-17F neutralizing antibodies (gray bars). Results are shown as mean ± SEM (n = 4–5) (*$p < 0.05$; **$p < 0.01$).

See also Figure S5.

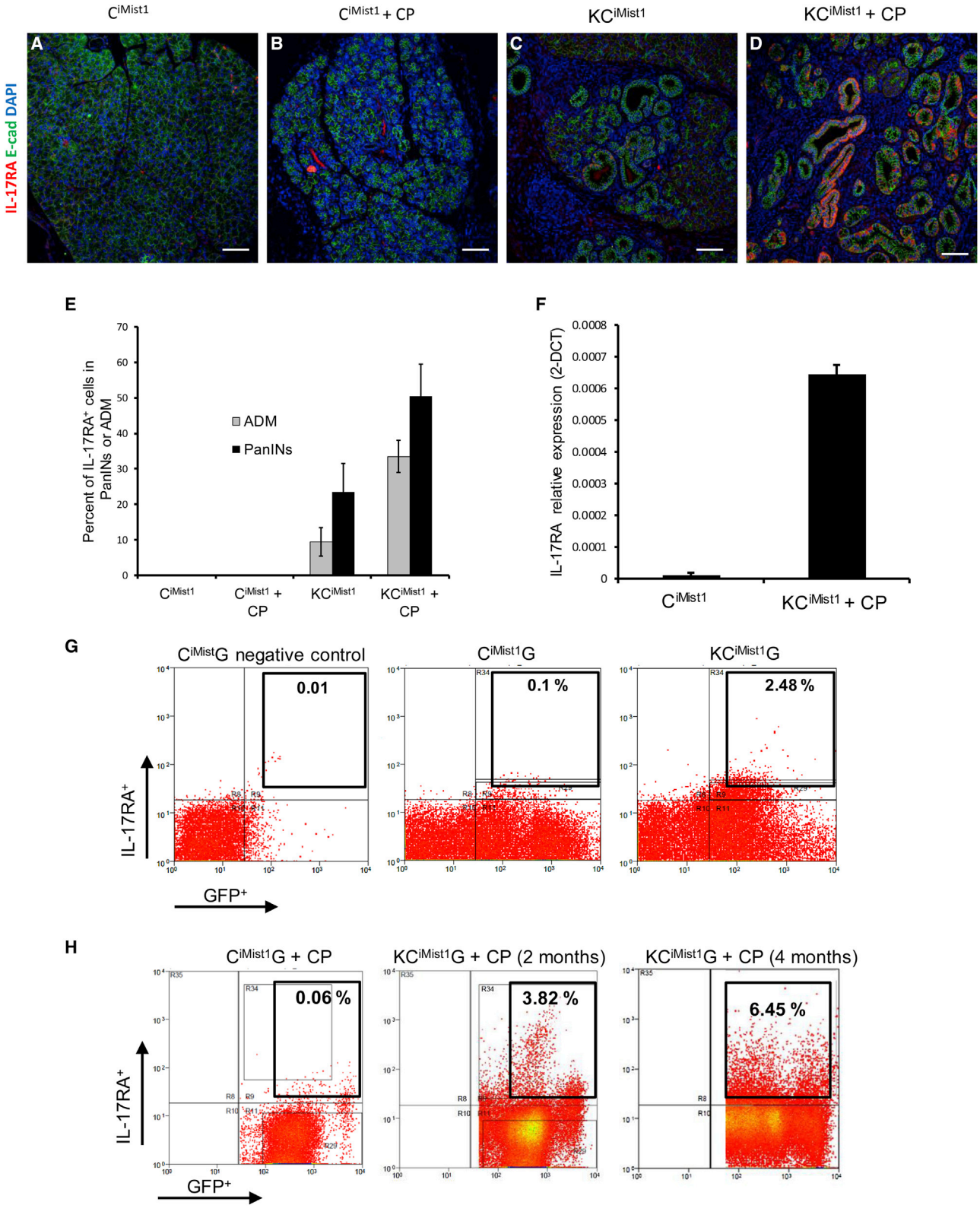

Figure 6. Oncogenic Kras Activates IL-17 Receptor A Expression in Early PanIN Epithelium

(A–D) Immunofluorescent detection of IL-17RA expression (red) on pancreatic epithelium in C^{iMist1} normal pancreas (A), C^{iMist1} pancreas with associated chronic pancreatitis (B), KC^{iMist1} pancreas at 8 weeks following Kras activation (C), and KC^{iMist1} with chronic pancreatitis at 6 weeks following Kras activation (D). Slides were colabeled with anti-E-cadherin antibody (green) and DAPI nuclear marker (blue). Red arrow indicates IL-17R staining in stroma. Scale bars represent 80 μm.

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analysis, multiple IL-17-related functional groups were found to be downregulated in mPanIN epithelial cells harvested from mice undergoing pharmacologic inhibition of IL-17 signaling, confirming a direct in vivo effect of IL-17 on IL-17 receptor-expressing mPanIN epithelial cells (Figure 7B). In examining individual genes, we found that multiple genes previously shown to be regulated by IL-17 in other systems were also significantly downregulated in mPanIN epithelial cells isolated from IL-17 neutralized mice, including *Cxcl5* (Liu et al., 2011), *Lcn2* (Shen et al., 2005), *Muc5ac* (Fujisawa et al., 2009), *Rgs13* (Xie et al., 2010), and *Il6* (Ogura et al., 2008; Wang et al., 2009) (Figures 7C and 8A; Table S1). Among these, *Muc5ac* and *Il6* have previously been associated with pancreatic tumor progression (Hoshi et al., 2011; Lesina et al., 2011). Other genes associated with pancreatic tumorigenesis but not previously known to be regulated by IL-17 were also found to be downregulated in the same cohort, including *Mmp7* (Fukuda et al., 2011), *Dclk1* (Bailey et al., 2013), *Muc4* (Chaturvedi et al., 2007; Singh et al., 2004), *Ctse* (Cruz-Monserrate et al., 2012), *Tff1* (Prasad et al., 2005), and *Onecut2* (Prévot et al., 2012) (Figure 7C; Table S1). Together, these findings implicate a direct hematopoietic-to-epithelial IL-17 signaling axis as a critical regulator of early pancreatic neoplasia.

Based on the known role of IL-6/Stat3 signaling in pancreatic neoplasia, we further investigated the association between loss of IL-17 signaling and downregulated IL-6 expression. Using qRT-PCR, we noted a 90% decrease in *Il6* expression in FACS-isolated mPanIN epithelial cells following even a single week of neutralizing antibody-based IL-17 signaling blockade (Figure 8A). A similar loss of *Il6* expression was observed in pancreatic tissue harvested from KC^{iMist1}/IL-17^{KO} BM mice (Figure 8B). Additional evidence that this decrease in IL-6 expression was functionally significant was provided by examination of Stat3 activation in mPanIN epithelial cells. Compared to mPanIN lesions arising in KC^{iMist1}/IL-17^{WT}BM mice, less abundant PanIN epithelial cells arising in KC^{iMist1}/IL-17^{KO} BM mice displayed a marked reduction in phospho-Stat3 (Figure 8C), consistent with loss of IL-17-dependent IL-6 expression.

DISCUSSION

The critical role of host immunity in regulating the early stages of tumorigenesis is well established (Grivennikov et al., 2010), and mounting evidence has demonstrated the frequent failure to mount an effective antitumor immune response within the tumor microenvironment. In pancreatic cancer, many alterations in the microenvironment appear to occur as a direct response to oncogenic Kras. Besides a cell-autonomous influence on pancreatic epithelial cells, epithelial Kras activation also induces dramatic stromal remodeling and expansion, including the recruitment of both proinflammatory and immunosuppressive cell populations. Prior work in the field has begun to elucidate the cellular and soluble components mediating interactions between neoplastic pancreatic epithelium and adjacent inflammatory cells. Among these, GM-CSF and other soluble factors have recently been implicated in tumor-induced expansion and recruitment of immunosuppressive cell populations (Bayne et al., 2012; Pylayeva-Gupta et al., 2012), while IL-8 and the IL-6-Stat3 pathway contribute to the induction of a protumorigenic inflammatory microenvironment (Fukuda et al., 2011; Lesina et al., 2011; Sparmann and Bar-Sagi, 2004). In turn, inflammatory cells and other stromal elements exert a potent effect on Kras-activated epithelial cells, as the induction of chronic pancreatitis accelerates the process of pancreatic tumorigenesis in both mice and humans.

Our work now implicates a hematopoietic-to-epithelial IL-17 signaling axis as another important driver of PanIN initiation and progression. Previous studies have demonstrated that IL-17A can elicit protumorigenic effects through a variety of mechanisms. In a spontaneous genetic model of prostate cancer, a prior report demonstrated that mice deficient of an IL-17 receptor expressed in prostatic tumorigenic epithelium had a reduced incidence of invasive prostate adenocarcinoma, indicating that IL-17 may promote the formation and growth of prostate adenocarcinoma (Zhang et al., 2012). In a different study using lymphoma, prostate and melanoma cell line xenografts, the absence of IL-17RA was associated with decreased tumor growth (He et al., 2010). Similarly, depletion of IL-17 not only decreased DMBA/TPA–induced inflammation and keratinocyte proliferation, but also delayed skin papilloma development (Xiao et al., 2009).

Although these studies and our results suggest that proinflammatory T_H17 cells accelerate early neoplasia, the effects of IL-17 on pancreatic cancer growth may be complex. Several transplantable tumor models have reported an anti-tumorigenic role (Garcia-Hernandez et al., 2010). With respect to pancreatic cancer, it has been reported that murine pancreatic cancer cells engineered to produce IL-6 display enhanced infiltration by T_H17 cells and reduced growth rates following subcutaneous injection into syngeneic mice, suggesting that T_H17 cells may retard pancreatic tumorigenesis (Gnerlich et al., 2010). However, functional studies of infiltrating T_H17 cells were not conducted in this study, and their increased recruitment may simply reflect known effects of IL-6. In addition, prior studies have correlated T_H17 cell infiltration and plasma IL-17A levels with poor prognosis in pancreatic cancer patients (He et al., 2011; Vizio et al., 2012).

(E) Quantification of IL-17RA expressing cells, expressed as percent of all E-cadherin$^+$ cells in ADM or PanIN lesions. Results are shown as mean ± SEM. Multiple lesions (ADMs and PanINs) from 5 mice were quantified (*$p < 0.05$; **$p < 0.01$).

(F) Relative expression of IL-17RA quantified by TaqMan RT-PCR on GFP$^+$ cells sorted from C^{iMist1} mice versus $KC^{iMist-1}$ mice. Results are shown as mean ± SEM ($n = 3$).

(G) FACS-based quantification of cells expressing cell surface IL-17RA in combination with GFP as a marker of effective Cre-based recombination in mice 8 weeks postoncogenic Kras activation. The "C^{iMist1}G negative control" panel depicts cells harvest from C^{iMist1}G control mice receiving no tamoxifen labeled with secondary antibody only; these cells were used to establish subsequently utilized GFP and IL-17RA gates. Propidium iodide staining was used to exclude nonviable cells.

(H) FACS-based quantification of cells expressing cell surface IL-17RA in combination with GFP following oncogenic Kras activation and CP. Propidium iodide staining was used to exclude nonviable cells. For clarity, only the GFP$^+$ fraction is depicted in the FACS plots. Results are expressed as percent of total GFP$^+$ cells. See also Figure S6.

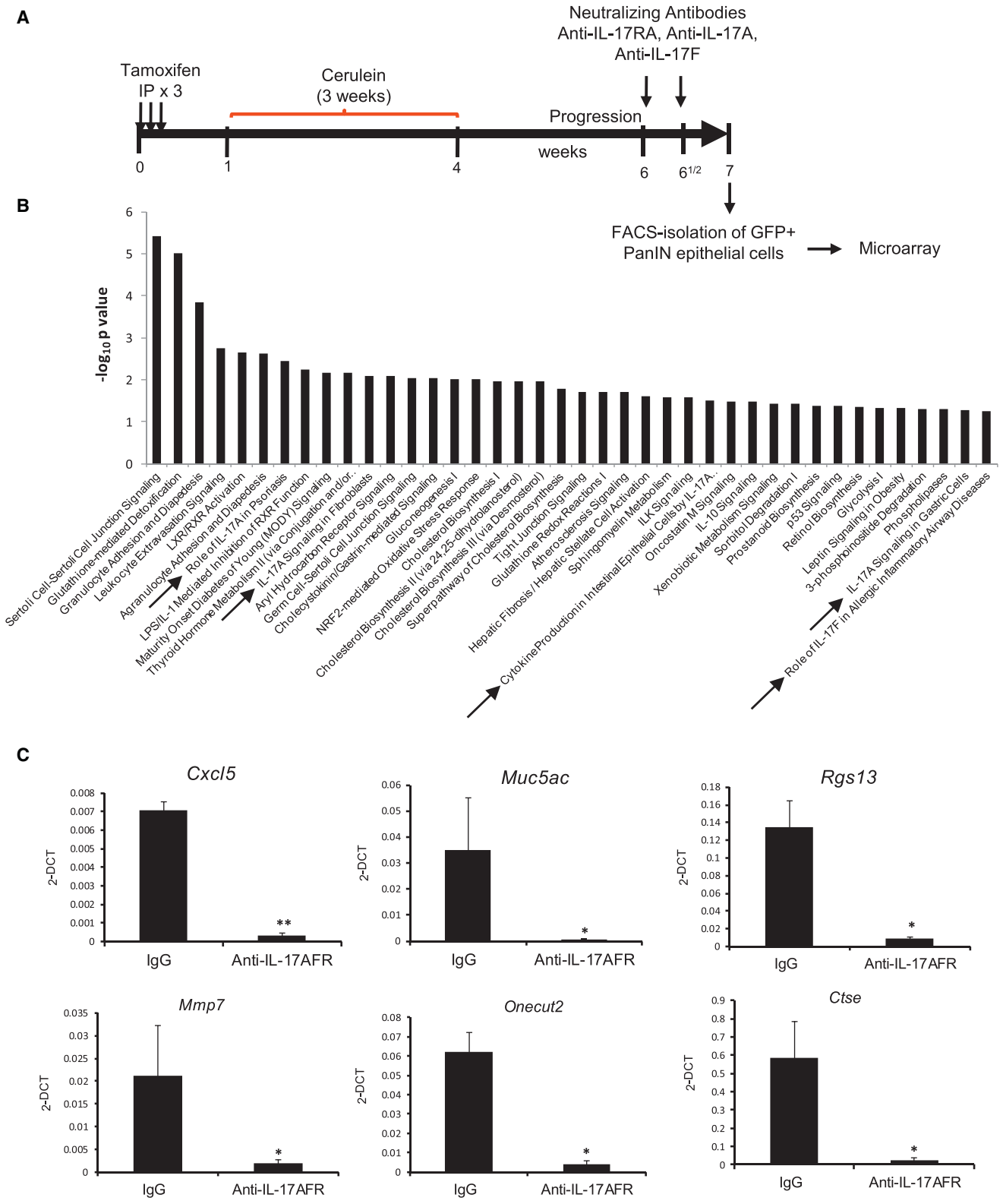

Figure 7. Kras^{G12D}-Induced Activation of IL-17RA Expression on PanIN Epithelial Cells Is Associated with In Vivo Functional Responses to IL-17A

(A) Protocol followed for induction of Kras^{G12D} expression in KC^{iMist1}G mice followed by delayed treatment with IL-17 signaling neutralizing antibodies and subsequent FACS-isolation of GFP^{+} PanIN epithelial cells for microarray.

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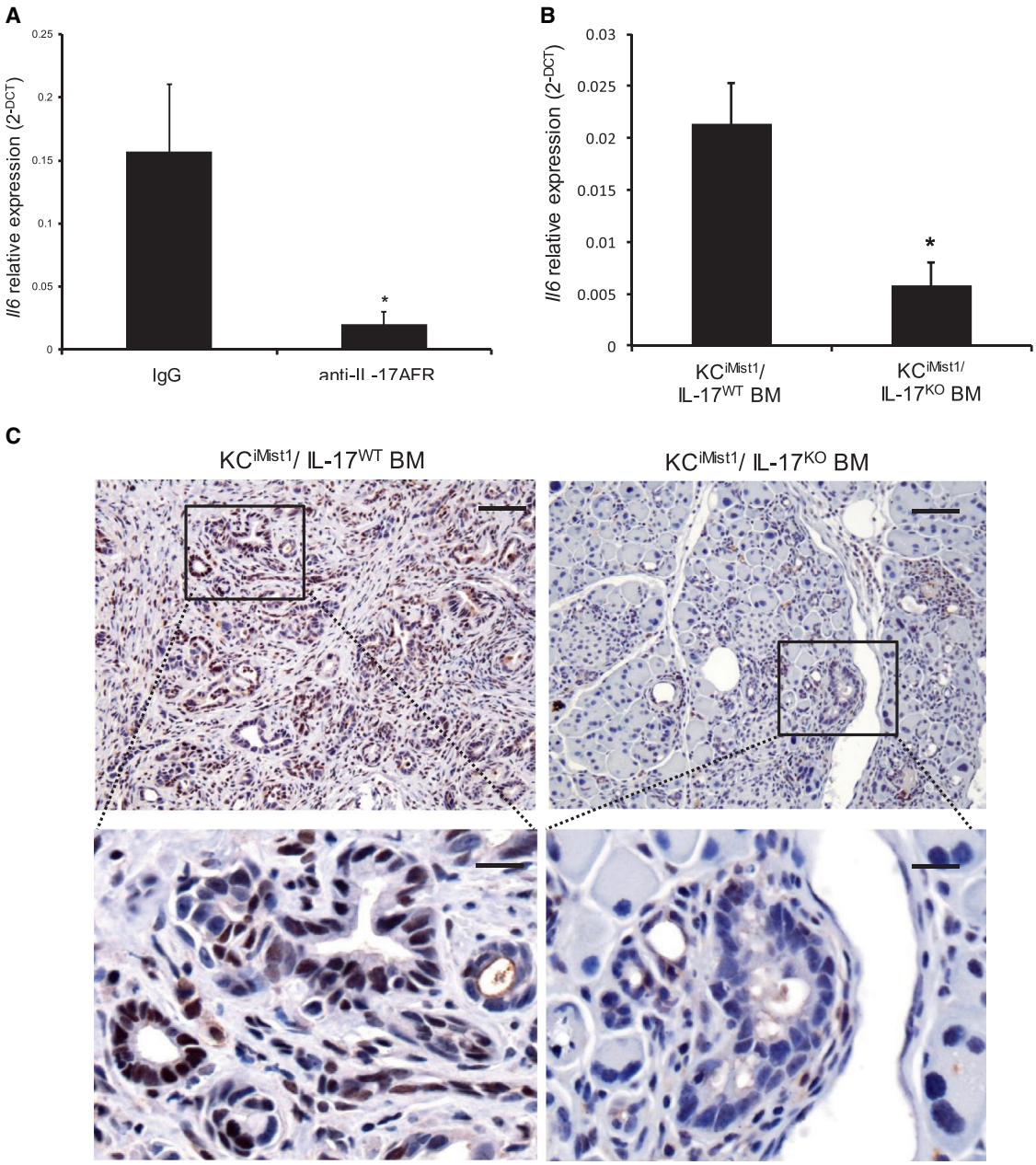

Figure 8. IL-17 Neutralization Is Associated with Decreased IL-6/p-Stat3 Epithelial Activation

(A) Relative expression of *Il6* in GFP$^+$ cells sorted from KC^{iMist1}G mice treated for 1 week with IL-17 signaling neutralizing antibodies. Results are shown as mean ± SEM (n = 4) (*$p < 0.05$; **$p < 0.01$).

(B) Relative expression of *Il6* in pancreatic whole tissue from KC^{iMist}/IL-17^{WT}BM versus KC^{iMist}/IL-17^{WT} mice. Results were presented as relative expression of *Il6* (2^{-DCT}). Results are shown as mean ± SEM (n = 4) (*$p < 0.05$).

(C) Immunohistochemistry for phosphorylated Stat3 (pStat3) in dysplastic pancreatic epithelium of KC^{iMist}/IL-17^{WT} BM versus KC^{iMist}/IL-17^{KO} mice (bottom panel). Scale bars in top panels represent 80 μm. Bottom panels are showing detailed dysplastic epithelium from top panels.

Nevertheless, it is clearly possible that IL-17 signaling may exert opposing influences on pancreatic tumorigenesis at different stages of the disease and in the context of variability in the host immune response. It should also be noted that many of our experiments were performed in the setting of concomitant inflammation, potentially amplifying the role played by IL-17.

(B) Top Ingenuity canonical pathways enriched among genes that were significantly downregulated in GFP$^+$ cells sorted from KC^{iMist1}G mice treated with IL-17 signaling neutralizing antibodies. As indicated on y axis, pathways are sorted based on p value. Arrows indicate pathways directly related to IL-17 signaling.

(C) Relative expression of representative genes quantified by TaqMan RT-PCR. Results are shown as mean ± SEM (n = 3) (*$p < 0.05$; **$p < 0.01$).

See also Table S1.

However, the fact that RORγt-expressing cells are also found in human PanIN, and the fact that oncogenic Kras can itself induce inflammation (Clark et al., 2007), suggest that our observations may indeed be broadly applicable.

When we analyzed IL-17-producing cell types associated with early pancreatic neoplasia, we found that both T_H17 cells and γδT cells were specifically recruited to the pancreatic preneoplastic microenvironment, with both cell types likely contributing similar amounts of IL-17. In addition to the roles of T_H17 cells described above, IL-17-producing γδT cells have been similarly implicated in chronic inflammatory conditions and autoimmune pathologies (Braun et al., 2008; Ito et al., 2009), as well as in tumor progression (Wakita et al., 2010). As we have not specifically studied the individual roles of T_H17 cells and γδT cells during PanIN formation, it is important to recognize that other hematopoietic cell types may in fact be important sources of IL-17; in this regard, our FACS analyses indicate that T_H17 cells and γδT cells together account for approximately 75% of all $CD45^+$, IL-17-expressing cells within the PanIN microenvironment.

Regardless of its source, our results suggest that IL-17 may, at least in part, exert its proneoplastic effects by direct interaction with IL-17 receptors on emerging PanIN epithelium, whose expression is activated in a cell-autonomous manner by oncogenic Kras. Using whole transcriptome analysis of FACS-isolated PanIN epithelial cells, we observe dramatic changes in previously defined IL-17-dependent gene expression signatures following even short term loss of IL-17 signaling, confirming the in vivo functionality of hematopoietic-to-epithelial IL-17 signaling in early pancreatic neoplasia. Among the genes displaying IL-17-dependent expression in IL-17 receptor-expressing PanIN epithelial cells was IL-6, previously identified as a critical driver of pancreatic neoplasia (Lesina et al., 2011). This decrease in epithelial IL-6 expression observed following IL-17 inhibition was associated with loss of phospho-Stat3 in PanIN epithelial cells. Although these data are merely correlative, they imply that IL-17 and IL-6-Stat3 signaling may cooperatively accelerate PanIN initiation.

In addition to direct effects of IL-17 on PanIN epithelial cells, we are aware of possible additional indirect effects. In this regard, it has also been shown that IL-17A can promote tumorigenesis by signaling through IL-17RA present on mesenchymal or endothelial cells (Numasaki et al., 2004; Takahashi et al., 2005), and this could represent an additional mechanism explaining the protumorigenic effect of IL-17 in our model. A recent report (Chung et al., 2013) further emphasizes the role of IL-17 in modulating the tumor vasculature by directing the effector function of MDSCs and in inducing fibroblast-mediated secretion of proinflammatory cytokines. It has been previously reported that IL-17 is required for the development of MDSCs in tumor-bearing mice, as a defect in IL-17RA reduces the number of tumor-infiltrating MDSCs (He et al., 2010). We have observed a significant decrease in MDSCs in the PanIN microenvironment after neutralizing IL-17 signaling, suggesting that another mechanism for IL-17's tumor-promoting effect might be through the differentiation and/or recruitment of MDSCs. As we have observed that IL-6 expression by pancreas-infiltrating MDSCs is dramatically activated in both KC^{iMist1} and KC^{iMist1} + CP mice (data not shown), this effect may also contribute to the loss of IL-6-Stat3 signaling we observe in the absence of IL-17.

In addition to studies in the mouse, we were also able to confirm that human PanIN lesions exhibit upregulated expression of IL-17RA and that human ADMs as well as PanINs are infiltrated by RORγt-expressing cells. Together, these studies identify a therapeutically targetable signaling axis of potential relevance in the treatment and/or prevention of pancreatic cancer.

EXPERIMENTAL PROCEDURES

Detailed materials and methods are provided in Supplemental Experimental Procedures.

Genetically Engineered Mice

All animal experiments were conducted in compliance with the National Institute of Health guidelines for animal research and approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University. A tamoxifen-inducible $Mist1^{CreERT2/+}$ (C^{iMist1}) driver strain was used to activate a conditional lox-stop-lox-$Kras^{G12D}$ allele, as previously described (Habbe et al., 2008). A cohort of KC^{iMist1} mice was also crossed onto a Cre-sensitive double fluorescent reporter line, $Rosa26^{mTmG}$ (Muzumdar et al., 2007). The resulting triple transgenic $Mist1^{CreERT2/+}$;LSL-$Kras^{G12D}$;$R26^{mTmG}$ ($KC^{iMist1}G$) mice enabled FACS-based isolation of $Kras^{G12D}$-expressing cells by virtue of simultaneous GFP activation.

Human Pancreatic Tissue Microarrays

Tissue microarrays containing wide range of normal pancreas, ADM and PanIN were constructed using paraffin tissue blocks. This study was Johns Hopkins University-Institutional Review Board (IRB) exempt as no protected health information was used.

Dissociation of Adult Mouse Pancreas

Whole adult mouse pancreas was harvested and digested in 1 mg/ml collagenase-P (Boehringer Mannheim) at 37°C for 30 min. Following multiple washes with Hank's balanced salt solution (HBSS) supplemented with 5% FBS, collagenase-digested pancreatic tissue was filtered through a 600 μm polypropylene mesh (Spectrum Laboratories) and spun down. The pellet was then diluted in trypsin (0.05%) (Mediatech) and incubated at 37°C for 5 min. After multiple washes, cells were finally filtered through a 100 μm cell strainer and directly resuspended in HBSS for flow cytometry.

Adenoviral Infection

Adenovirus (5×10^9 plaque-forming units [pfu] suspended in 50 μl) encoding either GFP (Ad-EGFP), Luciferase (Ad-Luc), or IL-17A (Ad-IL-17A) (Schwarzenberger et al., 1998) were injected directly into multiple sites throughout the pancreatic parenchyma. A cohort of mice was sacrificed 1 week following adenoviral injection to assess for expression of encoded genes, using fluorescent microscopy for detection of eGFP in mice injected with Ad-EGFP and ELISA for detection of IL-17A in mice injected with either Ad-IL-17A or Ad-Luc. A second cohort of mice was sacrificed at 7 weeks following Kras activation.

Neutralizing Antibody Administration

Monoclonal neutralizing antibodies against IL-17RA, IL-17A, and IL-17F were generously provided by Amgen (Sarkar et al., 2009; Stagg et al., 2011; Teng et al., 2012).

Bone Marrow Preparation and Transplantation

Femurs and tibias were harvested from sacrificed donor mice and the bone marrow was flushed with RPMI from bone canals into a Petri dish. The flushed marrow were then filtered through a 100 μm cells strainer. Cells were then spun and resuspended in sterile PBS at a concentration of 10,000,000 cells/200 ul. Recipient mice were irradiated with a total of 900 cGy and allowed to rest for 6 hr. Mice were then injected retro-orbitally with bone marrow cells

(10,000,000 cells/mouse) using an intradermal 26 G × 3/8 syringe. Mice were evaluated for effective bone marrow chimerism at 8 weeks after the transplant.

RNA Isolation and Gene Array Analysis

RNA was immediately isolated from GFP$^+$ sorted PanIN epithelial cells using the QIAGEN RNeasy extraction kit. Gene array analysis was performed using Mouse exon microarrays 1.0 ST (Affymetrix).

Statistical Analysis

Data are summarized as mean ± SEM. Data were analyzed using GraphPad Prism (GraphPad Software). Comparisons between groups where data were normally distributed were made with Student's t test, and comparisons among multiple groups or nonparametric data were made with ANOVA. Significance was accepted at a p value < 0.05.

ACCESSION NUMBERS

Microarray data has been deposited at Gene Expression Omnibus (accession number GSE54753).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2014.03.014.

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