CD4⁺ T Lymphocyte Ablation Prevents Pancreatic Carcinogenesis in Mice

Yaqing Zhang, Wei Yan, Esha Mathew, Filip Bednar, Shanshan Wan, Meredith A. Collins, Rebecca A. Evans, Theodore H. Welling, Robert H. Vonderheide, and Marina Pasca di Magliano

Abstract

Pancreatic cancer, one of the deadliest human malignancies, is associated with oncogenic Kras and is most commonly preceded by precursor lesions known as pancreatic intraepithelial neoplasias (PanIN). PanIN formation is accompanied by the establishment of an immunotolerant microenvironment. However, the immune contribution to the initiation of pancreatic cancer is currently poorly understood. Here, we genetically eliminate CD4⁺ T cells in the iKras⁺ mouse model of pancreatic cancer, in the context of pancreatitis, to determine the functional role of CD4⁺ T cells during mutant Kras-driven pancreatic carcinogenesis. We show that oncogenic Kras-expressing epithelial cells drive the establishment of an immunosuppressive microenvironment through the recruitment and activity of CD4⁺ T cells. Furthermore, we show that CD4⁺ T cells functionally repress the activity of CD8⁺ T cells. Elimination of CD4⁺ T cells uncovers the antineoplastic function of CD8⁺ T cells and blocks the onset of pancreatic carcinogenesis. Thus, our studies uncover essential and opposing roles of immune cells during PanIN formation and provide a rationale to explore immunomodulatory approaches in pancreatic cancer.

Introduction

Pancreatic ductal adenocarcinoma (PDA), the most common form of pancreatic cancer with one of the highest mortality rates among solid malignancies, is preceded by precursor lesions, the most common of which are known as pancreatic intraepithelial neoplasia (PanIN; ref. 1). PanIN are defined on the basis of characteristic changes in the epithelial cells, namely the formation of enlarged duct-like structures, with accumulation of intracellular mucin and progressive dysplasia (2). In addition to the epithelial changes, PanIN are accompanied by changes in the surrounding microenvironment, including the accumulation of cells with myofibroblast-like features, deposition of collagen-rich extracellular matrix, and infiltration of immune cells. Although abundant immune cells are a constant feature at all stages of pancreatic neoplastic progression (3), these cells are mostly immunosuppressive in nature, even at disease inception. Infiltrating cells include CD4⁺ regulatory T cells (Treg), macrophages, and myeloid-derived suppressor cells (MDSC; ref. 3). The immune system plays a dual role in cancer (4): immunosuppression might prevent cancer onset and growth (5, 6), but a growing body of evidence indicates that immune cell subsets can promote tumorigenesis (7–10). The immune system represents an attractive therapeutic target, as modulating its activity toward an antitumor function could complement traditional cancer treatment, which is notably ineffective in pancreatic cancer. However, the interaction between the immune system and cancer initiation and progression needs to be better understood. Here, we focus on the role of CD4⁺ T cells during PanIN formation and progression.

Study of the contribution of components of the immune system to pancreatic carcinogenesis is only possible in a system that preserves the intact tumor microenvironment and mimics the immune response in human patients. Genetically engineered mouse models that most closely resemble the progression of human pancreatic cancer are based on pancreas-specific expression of oncogenic Kras. Oncogenic Kras is not only expressed in the vast majority of human pancreatic cancers (11, 12), it is also found in a high percentage of PanIN (13, 14); studies in mice have validated the notion that the presence of mutant Kras is required to initiate pancreatic carcinogenesis (15, 16). Although most commonly used, the KC mouse model of PDA expresses oncogenic Kras during the earliest stages of pancreas development (15), a situation differs from the human patients, whose Kras mutations are believed to occur sporadically in adulthood. Therefore, we developed the iKras⁺ model with inducible expression of oncogenic Kras, which allows the modulation of the mutant protein in adult mice (17, 18). Activation of oncogenic...
Kras during pancreatic development is sufficient for the development of PanIN lesions over time (15). In contrast, activation of oncogenic Kras in the adult pancreas fails to elicit carcinogenesis, possibly indicating that the adult pancreatic tissue is less plastic and thus less susceptible to transformation. However, the synergy of oncogenic Kras expression and the induction of inflammation (namely chronic or acute pancreatitis) induces rapid and extensive PanIN formation (17, 19). These findings are consistent with chronic pancreatitis being one of the key risk factors for pancreatic cancer in humans (20, 21). Of note, even in animals with embryonic Kras expression, the induction of pancreatitis accelerates PanIN formation and increases its penetrance (22, 23).

In the current study, we used the iKras+ model to investigate the contribution of CD4+ T cells to pancreatitis-driven PanIN formation. For this purpose, we genetically eliminated CD4+ T cells by crossing a CD4 loss-of-function allele with iKras+ mice, and analyzed PanIN formation and progression over time. Our results indicate an essential role for CD4+ T cells to the onset of pancreatic carcinogenesis and provide a rationale for modulating the activity of the immune system as part of pancreatic cancer prevention and treatment.

Materials and Methods

Mouse strains

We generated iKras+;CD4+/− mice by crossing previously described triple-transgenic mice iKras+(p48-Cre;R26-rtTa-IRES-EGFP;TetO-KrasG12D); ref. 17) with CD4-deficient mice (B6.129S6-Cd4tm1Knw/J; The Jackson Laboratory). Combinations of single- or double-mutant littersmates 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 conducted in compliance with the University of Michigan Committee on Use and Care of Animals (UCUCA) guidelines.

Doxycycline treatment

iKras+ or iKras+;CD4+/− mice were treated with doxycycline (Sigma) to induce KrasG12D expression. Doxycycline was administered in the low-dose doxycycline chow (50 mg/kg) or in the drinking water at a concentration of 0.2 g/L in a solution of 5% sucrose and was refreshed every 3 to 4 days.

Pancreatitis induction

Acute pancreatitis was induced using the cholecystokinin agonist caerulein (Sigma) on de-identified slides as previously described (17, 24).

Histopathologic analysis

Histopathologic analysis was conducted by a pathologist (W. Yan) on de-identified slides as previously described (17, 24, 25).

Flow cytometry

Single-cell suspensions of spleen and pancreas were prepared as follows: Spleens were crushed and passed through a 40-μm cell strainer and treated with red blood cell (RBC) lysis buffer (eBioscience) to eliminate erythrocytes. Pancreata were minced using sterile scalpels, and 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% fetal calf serum (FCS) with the following antibodies: anti-CD45 (MCD4530; Invitrogen); anti-CD3 (clone 17A2), anti-CD4 (clone RM4–5), anti-CD86 (clone 53–67), anti-CD25 (clone PC61), anti-CD11b (clone M1/70), anti-F4/80 (clone BM8), anti-CD11c (clone HL3), anti–Gr-1 (clone RB6–8C5), anti–RO1Rt (clone Q31–378) anti–Foxp3 (clone FJK-16s; all from BD Pharmingen), and anti-interleukin (IL)-17A (clone eBio1B7B7). Flow cytometry studies were performed in a CyAn ADP Analyzer (Beckman Coulter), and data were analyzed with Summit 4.3 software.

Intracellular cytokine staining (IFN-γ)/CD107a assay

Single-cell suspensions of spleen and pancreas were prepared. Cells were mixed with anti-CD107a fluorescein isothiocyanate (FITC; 1:100) and plated in a 96-well round-bottomed plate with GolgiStop protein transport inhibitor (containing monensin; BD Biosciences) and either complete media alone or media containing anti-CD3 (0.5 μg/mL) and anti-CD28 (1 μg/mL) as a positive control for T-cell stimulation. Cells were incubated at 37°C for approximately 5 hours and then were washed and stained for surface molecules. Cells were fixed and permeabilized using a fixation/permeabilization kit (eBiosciences) followed by intracellular cytokine staining with anti–IFN-γ phycoerythrin (PE; clone XMG1.2; BD Biosciences; 1:50) prepared in permeabilization buffer for approximately 20 minutes at 4°C. Cells were analyzed on a MoFlo Astrio (Beckman Coulter) for IFN-γ and CD107a expression on CD4+ CD8− Gr-1− p48− cells, and data were analyzed with Summit 4.3 software.

Anti-PanIN CD8+ T-cell activity assay

Single-cell suspensions of fresh spleens and pancreata from PanIN-bearing mice and wild-type (wt) mice were prepared. GFP+ PanIN cells and CD8+ T cells were purified by flow cytometry and cocultured in 48-well plate at a ratio of 1:5 for 4 hours.

CD8+ T-cell depletion

For depletion of CD8+ T cells, anti-CD8 monoclonal antibody (mAb; BioXcell #BE0061g; 2.43; 200 μg/mouse) was injected intraperitoneally on days −2, −1, and 0, and then twice per week.

Statistical analysis

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

Detailed protocols and standard procedures are included in the Supplementary Data.

Results

CD4+ T cells are required for pancreatitis-driven PanIN formation

To interrogate the contribution of CD4+ lymphocytes to pancreatic carcinogenesis, we generated iKras+;CD4− mice (Fig. 1A) and compared their neoplastic progression with that
of their iKras littermates. Adult mice (4–6 weeks of age) were administered doxycycline to induce oncogenic Kras expression; then acute pancreatitis was induced using the cholecystokinin agonist caerulein as described previously (17). The animals were subdivided into cohorts of 3 to 10 mice per genotype and harvested at the indicated time points (Fig. 1B). Littermate control mice, either lacking Cre recombinase or oncogenic Kras expression, were included for comparison.
Histologic changes consistent with the induction of pancreatitis were evident in all three cohorts 1 day following caerulein injections (Fig. 1C), including infiltration of immune cells, edema of the tissue, and acinar damage such as acinar-ductal metaplasia (ADM). In control mice, the pancreata had undergone tissue repair within 1 week of the induction of pancreatitis; the cycle of damage and repair was accompanied by transient activation of the mitogen—activated protein kinase (MAPK) signaling pathway (data not shown) as described previously (26, 27). The expression of oncogenic Kras’ dramatically altered the repair process, diverting it toward accumulation of a fibrotic stroma, and prolonged ADM. PanIN (mostly PanIN1A-2) were evident as early as 3 weeks after the induction of pancreatitis, and progressed to a majority of PanIN2–3 by 17 weeks (Fig. 1C, middle row and histopathologic quantification in Fig. 1D). This process was accompanied by persistent activation of MAPK signaling (Supplementary Fig. S1A), accumulation of intracellular mucin (Supplementary Fig. S1A, inset), extracellular collagen (Supplementary Fig. S1B), and smooth muscle actin (SMA)—positive fibroblasts (Supplementary Fig. S1C).

The tissue damage in iKras⁺;CD4⁻/⁻ mice did not differ significantly from the tissue damage observed in control and iKras⁺ mice during the acute phase of pancreatitis (Fig. 1C, I-day time point). However, by 1 week following pancreatitis, we observed heterogeneity in the tissue, with some acini appearing histologically normal, but other areas demonstrating ADM surrounded by abundant inflammatory infiltrates (Fig. 1C, 1-week time point). At the 3-week-time point—when tissue-wide PanIN had replaced the iKras⁺ pancreatic parenchyma—evaluation of iKras⁺;CD4⁻/⁻ pancreata revealed mostly normal acini interspersed by ADM and rare PanIN1A-2. The prevalence of normal acini increased, whereas ADM/PanIN lesions decreased over time (Fig. 1C, 3 and 8 weeks and histopathologic quantification in Fig. 1D). By 17 weeks after the induction of pancreatitis, tissues in the iKras⁺;CD4⁻/⁻ mice were largely composed by acini of normal histology and occasional adipocyte infiltrates (Fig. 1C, 17 weeks, and quantification in Fig. 1D). Accordingly, p-ERK1/2 accumulation was sporadic and little to no periodic acid–Schiff (PAS) and Tri-chrome staining was observed in the iKras⁺;CD4⁻/⁻ tissues at any of the time points (Supplementary Fig. S1A and S1B), even though the expression of oncogenic Kras’ was unchanged (Supplementary Fig. S3). Moreover, the accumulation of SMA-positive fibroblasts—which previously we have shown to be dependent on epithelial oncogenic Kras expression—was reduced (Supplementary Fig. S1C). Thus, CD4⁺ T lymphocytes were required for the pancreatitis-driven PanIN formation, as well as for the establishment of a fibrotic microenvironment, which is a key feature of pancreatic cancer.

**ADM/PanIN cells in CD4-deficient mice undergo apoptosis**

Because iKras⁺ and iKras⁺;CD4⁻/⁻ tissues appear virtually indistinguishable 1 day after the induction of pancreatitis, but then follow divergent paths toward PanIN progression or tissue repair, we investigated the fate of ADM and early lesions, and of normal acini, in both cohorts of animals. In iKras⁺ mice, high proliferation—measured by Ki67 positivity—was observed both at the peak of pancreatitis-induced damage and in the lesions at all the later time points considered (Fig. 2A and C). High proliferation was also observed in iKras⁺;CD4⁻/⁻ pancreata 1 week after the induction of pancreatitis. However, in these animals, the burst of proliferation was transient, confined to the acinar cells, and likely corresponded to the repair of the acinar compartment. In contrast, in the rare lesions persisting at later time points, the epithelium was largely Ki67 negative. Previously, we have described how the presence of active, oncogenic Kras in the pancreatic epithelium mediates the formation and maintenance of an active, highly proliferative stroma (17). Consistently, we observed elevated proliferation both in the epithelial compartment and in the stroma in iKras⁺ pancreata at all time points analyzed (Fig. 2A and C, see quantification for compartment-specific proliferation). In iKras⁺;CD4⁻/⁻ mice, the proliferation in the stroma compartment was lower at all time points analyzed (Fig. 2A and C), thus explaining the lack of accumulation of collagen (Supplementary Fig. S1B) and the reduction in SMA positivity (Supplementary Fig. S1C).

We then measured the levels of apoptosis by cleaved caspase-3 immunostaining and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay. Rare cleaved caspase-3–positive cells were observed in iKras⁺ mice 3 weeks after the induction of pancreatitis and at later time points. In iKras⁺;CD4⁻/⁻ mice, ADM/PanIN lesions were rare; within those ADM/PanIN lesions, most epithelial cells expressed cleaved caspase-3 (Supplementary Fig. S2A). We then stained the tissue for TUNEL and CK19 and observed an increase of apoptosis in both the epithelial and the stroma compartments in iKras⁺;CD4⁻/⁻ mice compared with iKras⁺ mice (Fig. 2B and Supplementary Fig. S2B and quantification in Fig. 2D). Thus, in iKras⁺;CD4⁻/⁻ mice, the induction of pancreatitis led to rare ADM and PanIN that were unable to proliferate, underwent massive cell death, and were rapidly cleared from the tissue.

**Kras-dependent modulation of the inflammatory microenvironment requires CD4⁺ T cells**

The expression of oncogenic Kras dramatically altered the dynamics of immune infiltration in the pancreas following the induction of pancreatitis. One day after pancreatitis induction, both control littermates and iKras⁺ mice had abundant leukocytic infiltration (CD45⁺ cells: Fig. 3A). However, the nature of infiltrating cells was different, with a reduction in CD3⁺ T cells (especially the CD8⁺ subsets; Fig. 3B–D) and possibly an increase in Gr-1⁺ CD11b⁺ immature myeloid cells in iKras⁺ mice compared with control animals (Fig. 3G–I). One week after pancreatitis induction, the overall immune infiltration was greatly reduced in control mice—as expected on the basis of the histologic findings—while in iKras⁺ mice it remained elevated (Fig. 3A). The persisting immune cells included abundant Tregs (Fig. 3E), macrophages (Fig. 3F), and immature myeloid cells (Fig. 3G–I), but not CD8⁺ T cells (Fig. 3C). Moreover, both 1 day and 1 week after the induction of pancreatitis, a larger proportion of the CD4⁺ T cells were FoxP3 positive, and thus were Tregs, in the iKras⁺ mice than in
the control animals (Fig. 4A). In addition, low but detectable numbers of Th17 cells (IL-17A⁺; RORγt⁺) were detected in the control and iKras⁺ pancreata both 1 day and 1 week after pancreatitis induction (Fig. 4B), and there was an increased proportion of the IL-17⁺-positive subset of CD4⁺ T cells at 1 week compared with 1 day in the iKras⁺ pancreata. Their
relative numbers, as well as the expression of IL-17, were higher in iKras<sup>+/−</sup> mice than in control animals 1 week after pancreatitis induction. The Tregs were frequently closely associated with the nascent PanIN lesions, as revealed by immunostaining for FoxP3 (Fig. 4C). The percentage of Tregs remained unchanged in iKras<sup>+/−</sup> mice at 3 weeks, but could not be compared with that of control animals at this point as the latter had very few infiltrating cells left. Accordingly, several cytokines with protumor activity, such as granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-10, were upregulated in iKras<sup>+/−</sup> mice compared with control starting 1 day after the induction of pancreatitis (Supplementary Fig. S4A), and this effect persisted at later time points (Supplementary Fig. S4B). These findings are consistent with the establishment of an immunosuppressive microenvironment in Kras<sup>+/−</sup>-driven PanIN lesions, possibly regulated by different CD4 T-cell subsets.

Finally, we investigated the changes to the inflammatory microenvironment associated with a lack of CD4<sup>+</sup> T cells in wt and iKras<sup>+/−</sup> mice. CD4<sup>−/−</sup> mice presented with similar histologic findings as wt mice 1 day after pancreatitis induction (Supplementary Fig. S3A), but presented with persistent inflammation and areas of CK19-positive ADM 1 week later, when control animals had undergone complete tissue repair (Supplementary Fig. S3A and S3B). As expected, all CD4<sup>+</sup> T-cell subtypes were absent in CD4<sup>−/−</sup> mice, while total immune infiltrates (CD45<sup>+</sup> cells) and CD8<sup>+</sup> T cells remained evident at 1 week compared with control animals (Supplementary Fig. S3C–S3E). Thus, the lack of CD4<sup>+</sup> T cells causes a delayed tissue repair, accompanied with inflammatory infiltrates, in Kras wt mice following pancreatitis induction. In contrast, in iKras<sup>+/−</sup>;CD4<sup>−/−</sup> mice, the CD45<sup>+</sup> inflammatory infiltrates were abundant at 1 day, but rapidly decreased

Figure 3. Characterization of pancreatic immune infiltrates. Pancreatic immune cell infiltrates at 1 day and 1 week following pancreatitis induction were measured by flow cytometry to determine the percentage of CD45<sup>+</sup> leukocytes (A), CD3<sup>+</sup> T cells (B), CD3<sup>+</sup>CD8<sup>+</sup> T cells (C), CD3<sup>+</sup>CD4<sup>+</sup> T cells (D), CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs (E), CD11b<sup>+</sup>F4/80<sup>+</sup> macrophages (F), CD11b<sup>+</sup>Gr-1<sup>+</sup> (G), and CD11b<sup>+</sup>Gr-1<sup>−/−</sup> immature myeloid cells. Data, mean ± SEM; the statistical difference between experimental mice was determined by Student t tests. *P < 0.05; **P < 0.01; ***P < 0.001.
compared with iKras+/C3 mice (see 1 week, Fig. 3A). Thus, the lack of CD4+ T cells in the context of a Kras mutation caused a decrease in inflammatory cell infiltration. Further characterization confirmed that in iKras+/CD4+/C0;CD4+/C0 mice, largely due to the relative percentage increase in CD8+ T cells in the absence of CD4+ T cells (Fig. 3B and C). In addition, by 1 week after the induction of pancreatitis, the reduction of total leukocyte cells (CD45+ cells) was possibly due to the reduced infiltration of CD11b+F4/80+ macrophages and CD11b+Gr-1low and CD11b+Gr-1high cells in iKras+/CD4+/C0 mice compared with iKras+ mice (Fig. 3F–I).

Figure 4. Intrapancreatic Treg, Th17, and CD8+ T-cell infiltrations during PanIN formation. A, percentage of Tregs out of CD3+ CD4+ T cells in control and iKras+ pancreata 1 day, 1 week, and 3 weeks after pancreatitis induction. B, percentage of Th17 cells out of CD3+ CD4+ T cells in control and iKras+ pancreata 1 day and 1 week after pancreatitis induction. Data, mean ± SEM; the statistical difference between experimental mice was determined by Student t tests. *, P < 0.05; **, P < 0.01; ***, P < 0.001. C, FoxP3 immunohistochemistry staining in control, iKras+, and iKras+/CD4+/C0 pancreata 1 day, 1 week, and 3 weeks following pancreatitis induction. Data, mean ± SEM; n = 3 mice per cohort. The statistical difference between experimental mice was determined by Student t tests. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. D, quantification of CD8+ cells in control, iKras+, and iKras+/CD4+/C0 pancreata 1 day and 3 weeks following pancreatitis induction. Data, mean ± SEM; n = 3 mice per cohort. The statistical difference between experimental mice was determined by Student t tests. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. E, CD8 immunohistochemistry staining (scale bar, 25 μm). HPF, high-power field.
In addition, the expression of several cytokines was reduced in iKras<sup>+/−</sup>/CD4<sup>+</sup> pancreata compared with iKras<sup>+</sup>. Notably, the expression of the cytokines that are principally secreted by CD4<sup>+</sup> T-cell helper subsets (IL-2, IL-4, and IL-17) and the cytokines secreted by Tregs or MDSCs (IL-10 and IL-11) was significantly downregulated. In addition, there was a reduction in GM-CSF, a cytokine previously demonstrated in models of pancreatic neoplasia to enforce MDSC infiltration into PanIN and pancreatic tumors (28, 29). Other inflammatory cytokines secreted mostly by macrophages (IL-1β, IL-6, and IL-23) were also reduced in iKras<sup>+/−</sup>/CD4<sup>+</sup> pancreata compared with iKras<sup>+</sup> at this time point (Supplementary Fig. S4A). The down-regulation of this panel of cytokines was maintained over time, as indicated by quantitative real-time PCR (qRT-PCR) analysis of cDNAs derived from iKras<sup>+</sup> and iKras<sup>+/−</sup>/CD4<sup>+</sup> pancreata harvested 3 to 17 weeks after pancreatitis induction (Supplementary Fig. S4B).

Taken together, these results show that the nature of the immune cells infiltrating the pancreas is regulated by signals derived from oncogenic Kras-expressing epithelial cells, mediated by the action of CD4<sup>+</sup> T cells. Elimination of CD4<sup>+</sup> T cells reverses several of the Kras-driven changes in the inflammatory microenvironment of the pancreas. Tregs are a significant subset of CD4<sup>+</sup> T cells in Kras-mutant pancreata, and might be a key player during PanIN formation.

**Increase in intrapancreatic CD8<sup>+</sup> T-cell numbers and activity in iKras<sup>−/−</sup>/CD4<sup>−/−</sup> mice**

We then further explored the observation that CD8<sup>+</sup> T lymphocytes were increased in iKras<sup>+/−</sup>/CD4<sup>−/−</sup> compared with iKras<sup>+</sup> mice 1 day after the induction of pancreatitis. First, we performed immunostaining for CD8 in iKras<sup>+</sup> and iKras<sup>−/−</sup>/CD4<sup>−/−</sup> tissues 1 day, 1 week, and 3 weeks after the induction of pancreatitis (Fig. 4E). Our analysis of iKras<sup>+</sup> samples revealed very few CD8<sup>+</sup> cells within the pancreas, reflecting previous observations that CD8<sup>+</sup> T-cell infiltration is low during the onset of pancreatic cancer (3). In contrast, CD8<sup>+</sup> T cells were observed in iKras<sup>−/−</sup>/CD4<sup>−/−</sup> samples and often localized to the ADM/PanIN lesions (Fig. 4E and quantification in Fig. 4D). To measure the basal and stimulated effector function of CD8<sup>+</sup> T cells with the presence of other cell types in the tumor environment, we derived single-cell suspensions from the pancreata and *in vitro* cultured them with or without anti-CD3/CD28 stimulation. Then, we used flow cytometry to measure IFN-γ production as well as the expression of the activation marker CD107a in CD8<sup>+</sup> T cells. There was no difference in IFN-γ production or CD107a expression in CD8<sup>+</sup> T cells with or without stimulation between the iKras<sup>+</sup> and iKras<sup>−/−</sup>/CD4<sup>−/−</sup> cohorts at 1 day after pancreatitis induction (Fig. 5A). However, at the 1-week time point, the percentage of CD107α-expressing cells and CD107α IFN-γ cells in CD8<sup>+</sup> T cells was higher in the unstimulated samples derived from the iKras<sup>−/−</sup>/CD4<sup>−/−</sup> pancreata (Fig. 5B). At the 3-week time point, the percentage of IFN-γ-producing cells was higher in iKras<sup>−/−</sup>/CD4<sup>−/−</sup>-derived CD8<sup>+</sup> T cells, either with or without anti-CD3/CD28 stimulation; CD107α expression followed a similar trend, although the data did not reach statistical significance (Fig. 5C). Thus, these results indicate that CD8<sup>+</sup> T cells derived from the iKras<sup>−/−</sup> pancreata at 1- and 3-week time points are functionally inhibited in the presence of CD4<sup>+</sup> T cells.

These observations led us to hypothesize that a PanIN-specific immune response—driven by PanIN-specific CD8<sup>+</sup> T cells—occurred in iKras<sup>−/−</sup>/CD4<sup>−/−</sup> mice to block PanIN progression. To investigate this possibility, we isolated by flow cytometry both GFP<sup>+</sup> PanIN cells (EGFP linked to rtTa expression in the R26-rtTa transgene; see Fig. 1A) from iKras<sup>+</sup> and iKras<sup>−/−</sup>/CD4<sup>−/−</sup> and CD3<sup>+</sup> CD8<sup>+</sup> T cells from both the pancreata and the spleen of the same animals. CD3<sup>+</sup> CD8<sup>+</sup> T cells derived from wt spleen were used as control. We cocultured T cells with multiple PanIN cell lines derived from multiple mice. Four hours following coculture, we found a significant increase in both IFN-γ and granulocyte B production in CD8<sup>+</sup> T cells derived from the iKras<sup>−/−</sup>/CD4<sup>−/−</sup> pancrea compared with the T cells derived from either iKras<sup>+</sup> pancreata or iKras<sup>−/−</sup>/CD4<sup>−/−</sup> spleen (Fig. 5D). In contrast, CD8<sup>+</sup> T cells derived from the iKras<sup>−/−</sup> pancreata failed to upregulate expression of either marker following coculture with PanIN cells. Thus, tumor-specific CD8<sup>+</sup> T cells are present in iKras<sup>−/−</sup>/CD4<sup>−/−</sup> pancrea and react against PanIN cells.

On the basis of these findings, we hypothesized that CD8<sup>+</sup> T cells might mediate an antitumor response that is normally dampened by the immunosuppressive function of CD4<sup>+</sup> T cells.

**CD8<sup>+</sup> T-cell depletion rescues PanIN formation in iKras<sup>−/−</sup>/CD4<sup>−/−</sup> mice**

To determine whether the CD4<sup>+</sup> T-cell loss dampens neoplastic progression by enabling CD8<sup>+</sup> T-cell effector function, we sought to inhibit CD8<sup>+</sup> T cells and examine whether PanIN progression was restored. We depleted CD8<sup>+</sup> T cells using anti-CD8 mAb after administering doxycycline and before inducing pancreatitis with caerulein in a cohort of iKras<sup>−/−</sup>/CD4<sup>−/−</sup>; a second cohort of mice received control IgG, doxycycline, and caerulein. The animals were sacrificed 3 weeks after the induction of pancreatitis (Fig. 6A). By flow cytometry, we verified the successful depletion of CD8<sup>+</sup> T cells in the pancreata and spleen. We observed that pancreatitis fully resolved as expected in control animals at this time point, whereas in iKras<sup>−/−</sup> mice, most of the pancreatic parenchyma was replaced by ADM and PanIN lesions with only rare acini. As expected, tissue examination demonstrated that CD8<sup>+</sup> T cells in iKras<sup>−/−</sup> mice were largely absent in iKras<sup>−/−</sup>/CD4<sup>−/−</sup> mice depleted of CD8<sup>+</sup> T cells, whereas CD8<sup>+</sup> T cells were notable adjacent to neoplastic lesions in the iKras<sup>−/−</sup>/CD4<sup>−/−</sup> mice (Fig. 6D). Our
Figure 5. Enhanced CD8+ T-cell activity and PanIN-specific CD8+ T cells in iKras+/CD4+/+ mice. A–C, intracellular cytokine staining (IFN-γ)/CD107a assay. IFN-γ and CD107a expression in CD45(+CD8+CD11b–CD11c–CD4–CD19–) cells derived from control, iKras+, and iKras+/CD4+ pancreata was analyzed with flow cytometry at 1 day (A), 1 week (B), and 3 weeks (C) following pancreatitis induction. D, anti-PanIN CD8+ T-cell activity assay. GFP+ PanIN cells and CD3+CD8+ T cells derived from PanIN-bearing mice (pancreata and spleen) were purified by flow cytometry, and then cocultured for 4 hours. The expression of Ifnγ, Gzmb, and Prf-1 was analyzed by qRT-PCR. CD3+CD8+ T cells derived from wt spleen were used as control. Data, mean ± SEM; each point indicates one sample. The statistical difference was determined by Student t tests. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
findings indicate that CD4 depletion has no effect on carcinogenesis unless CD8+ T cells are present and, therefore, able to mediate an antineoplastic effect. In other words, CD8+ T cells have the potential ability to block the onset of pancreatic carcinogenesis, but seem hindered from doing so by the inhibitory function of CD4+ T cells.

**Discussion**

The goal of the current study was to determine the functional role of CD4+ T cells during mutant Kras-driven pancreatic carcinogenesis. By using a genetic system to eliminate CD4+ T cells in the iKras' mouse model of pancreatic cancer in the context of pancreatitis, we show that CD4+ T cells are required for the formation and progression of PanIN lesions, the most common precursor to pancreatic cancer. We further show that CD4+ T cells contribute to pancreatic carcinogenesis by blocking the antitumor activity of CD8+ T lymphocytes (Fig. 7A and B). Thus, ablation of CD8+ T cells rescues PanIN formation in CD4+ T cell–deficient iKras' mice (Fig. 7C). Our findings indicate that a productive immune response has the potential to block the onset of pancreatic cancer, but it is usually thwarted by immunomodulatory activity driven by mutant Kras-expressing precancerous cells and cross-talk with CD4+ T cells. Our data support the development of immunotherapeutic approaches to pancreatic cancer, and complement previous studies pioneering this concept (32–35).
The genetic approach we used in this study eliminated all the subsets of CD4\(^+\) T cells. Our results, therefore, lead to speculations about which CD4\(^+\) T-cell subset(s) may play the immunosuppressive role for which we provide evidence. Although Tregs are the most abundant CD4\(^+\) cell type in our system, Th17 cells accumulate in a Kras-dependent manner. Moreover, recent studies have highlighted the potential immunosuppressive role of Th2-skewed CD4\(^+\) T cells both in mice (34) and in humans (35). Elucidating the specific contribution of individual CD4\(^+\) T-cell subsets to tumorigenesis will be the next step in the characterization and understanding of the contribution of immune cells to pancreatic carcinogenesis.

Oncogenic Kras alters the immune response to pancreatitis

Previous studies have shown that the formation of PanIN lesions in mice bearing oncogenic Kras is accompanied by the infiltration of immune cells (3). Here, we have analyzed the immune infiltration following the induction of acute pancreatitis in wt mice and mice expressing oncogenic Kras. We found that both cohorts had extensive infiltration of immune cells during the peak damage induced by pancreatitis. However, significant differences were observed in both the nature and the duration of the immune response. First, in wt mice, the immune infiltration was transient and resolved within 1 week. In contrast, in iKras\(^+\) mice, the immune infiltration was sustained over time. Second, 1 day after the induction of pancreatitis, corresponding to peak damage and when the total number of immune cells was comparable between the two genotypes, iKras\(^+\) mice had fewer T cells but more macrophages and immature myeloid cells than wt animals. In addition, the expression of several cytokines, including IL-6, IL-10, and GM-CSF, was increased in iKras\(^+\) mice. Of note, IL-6 expression depends on sustained expression of oncogenic Kras in our model (36). Our results show that following tissue damage, the immune response is dramatically altered in the presence of oncogenic Kras, and parallels previous findings in the KC model upon spontaneous PanIN formation (3, 28, 29, 37, 38). Thus, signals derived from oncogene-expressing epithelial cells affect the surrounding microenvironment. We had previously shown that maintenance of an active population of tumor-associated fibroblasts depends on epithelial signals (17); here, we extend this observation to the infiltrating immune cells.

**CD4\(^+\) T cells are required to block CD8\(^+\) T cell–mediated antitumor immune responses**

The persistence of immune infiltration in oncogenic Kras-bearing mice compared with wt mice underlies the profound differences in the tissue repair process. Of note, wt mice undergo rapid tissue repair following acute pancreatitis, and the pancreas resumes its normal histologic appearance within 1 week following the induction of damage. In contrast, oncogenic Kras blocks the repair process and favors sustained ADM that leads to PanIN formation (22). At the same time, the lesions are surrounded by activated fibroblasts and immune cells. The nature of the immune infiltrates during the establishment of sustained ADM and PanIN formation suggests an immunosuppressive environment, with low representation of...
CD8⁺ T cells and abundant immunosuppressive leukocytes, including regulatory CD4⁺ T cells and immature myeloid cells (3). Here, we show that the Kras-driven establishment of an immunosuppressive environment, and in particular the exclusion of CD8⁺ T cells from the neoplastic area, requires CD4⁺ T cells. Thus, in the absence of CD4⁺ T cells, CD8⁺ T cells are found in the proximity of the developing lesions. Moreover, CD8⁺ T-cell effector functions were higher in the microenvironment of iKras⁺/CD4⁻/⁻ mice compared with iKras⁺ mice. Most importantly, depletion of CD8⁺ T cells rescued carcinogenesis in iKras⁺/CD4⁻/⁻ mice.

Our findings further show that suppression of CD8⁺ T cells underpins the carcinogenesis-promoting ability of CD4⁺ T cells. When CD8⁺ T cells were eliminated in iKras⁺/CD4⁻/⁻ mice, carcinogenesis was rescued, including not only PanIN formation but also the accumulation of a desmoplastic stroma. CD8⁺ T cells have been described as an essential antitumor component in pancreatic cancer (28, 29, 37). The ability of CD8⁺ T cells to block PanIN formation might be mediated by direct elimination of the preneoplastic cells. In fact, extensive apoptosis was observed in the lesions in iKras⁺/CD4⁻/⁻ animals, leading eventually to the elimination of the lesions from the tissue. A second aspect to be considered is the effect of CD8⁺ T cells on the stroma. Our results show that the accumulation of a fibroinflammatory stroma is inhibited by the absence of CD4⁺ T cells and rescued upon CD8⁺ T-cell depletion. Several interpretations are possible of this finding. CD8⁺ T cells might directly kill components of the stroma, and indeed we observed an increase in apoptosis in this compartment. However, we have shown previously that proliferation, activation, and survival of the stroma depend on epithelial signals (17); thus, it is possible that the lack of stroma in iKras⁺/CD4⁻/⁻ mice is a consequence of the lack of epithelial neoplasia.

It is interesting to note that CD4⁺ T cells have been studied in many tumor types with different results. In a PyMT model of breast cancer, CD4⁺ T cells were dispensable for the onset of carcinogenesis and the growth of the primary tumor, but essential for the establishment of metastases through their ability to modulate the function of myeloid cells (9), and independently from the presence of CD8⁺ T cells. In MYC- and BCR-ABL–based mouse models of T-cell acute lymphoblastic lymphoma and pro-B-cell leukemia, respectively, CD4⁺ T cells were dispensable for tumor growth but essential for tumor regression upon oncogene inactivation (39). Thus, the role of CD4⁺ T cells and their interaction with CD8⁺ T cells during the onset of carcinogenesis is exquisitely tissue specific.

Disclosure of Potential Conflicts of Interest
R.H. Vonderheide has received commercial research support from Pfizer and Roche and is a consultant/advisory board member for Genentech and Clovis. No potential conflicts of interest were disclosed by the other authors.

Disclaimer
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the article.

Authors’ Contributions
Conception and design: Y. Zhang, M.P. di Magliano
Development of methodology: Y. Zhang, R.A. Evans, R.H. Vonderheide
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Zhang, W. Yan, E. Mathew, P. Bednar, S. Wan, M.A. Collins
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Zhang, W. Yan, E. Mathew, P. Bednar, S. Wan, R.A. Evans, T.H. Welling, R.H. Vonderheide, M.P. di Magliano
Writing, review, and/or revision of the manuscript: Y. Zhang, M.A. Collins, R.A. Evans, T.H. Welling, R.H. Vonderheide, M.P. di Magliano
Study supervision: M.P. di Magliano

Acknowledgments
The authors thank Arthur L. Brannon III and Dr. Jörg Zeller for scientific discussion and critical reading of the article. The authors also thank Marsha M. Thomas for laboratory support. The p48-Cre mouse was a generous gift from Dr. Chris Wright (Vanderbilt University). The Ck19 antibody (Tromba III) was obtained from the Iowa Developmental Hybridoma Bank.

Grant Support
This project was supported by the University of Michigan Biological Scholar Program, the University of Michigan Cancer Center, and NC1-IR01CA151588-01. M.A. Collins was supported by a University of Michigan Program in Cellular and Molecular Biology training grant (NH T22 GM07315) and by a University of Michigan Center for Organogenesis Training Grant (5-T32-HD007515). R.H. Vonderheide and R.A. Evans were supported by NIH grant ROI CA169123.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 28, 2014; accepted January 31, 2014; published OnlineFirst February 11, 2014.

References
CD4⁺ T Cells Promote Pancreatic Cancer


CD4+ T Lymphocyte Ablation Prevents Pancreatic Carcinogenesis in Mice

Yaqing Zhang, Wei Yan, Esha Mathew, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/2326-6066.CIR-14-0016-T

Supplementary Material
Access the most recent supplemental material at:
http://cancerimmunolres.aacrjournals.org/content/suppl/2014/02/11/2326-6066.CIR-14-0016-T.DC1

Cited articles
This article cites 39 articles, 17 of which you can access for free at:
http://cancerimmunolres.aacrjournals.org/content/2/5/423.full#ref-list-1

Citing articles
This article has been cited by 8 HighWire-hosted articles. Access the articles at:
http://cancerimmunolres.aacrjournals.org/content/2/5/423.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerimmunolres.aacrjournals.org/content/2/5/423.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.