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The triterpenoid CDDO-imidazolide reduces immune cell infiltration and cytokine secretion in the Kras^{G12D};Pdx1-Cre (KC) mouse model of pancreatic cancer

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Abstract

Because the 5-year survival rate for pancreatic cancer remains under 10%, new drugs are needed for the prevention and treatment of this devastating disease. Patients with chronic pancreatitis have a 12-fold higher risk of developing pancreatic cancer. LSL-Kras$^{G12D/+}$;Pdx-1-Cre (KC) mice replicate the genetics, symptoms and histopathology found in human pancreatic cancer. Immune cells infiltrate into the pancreas of these mice and produce inflammatory cytokines that promote tumor growth. KC mice are particularly sensitive to the effects of lipopolysaccharide (LPS), as only 48% of KC mice survived an LPS challenge while 100% of wildtype (WT) mice survived. LPS also increased the percentage of CD45+ immune cells in the pancreas and immunosuppressive Gr1+ myeloid-derived suppressor cell in the spleen of these mice. The triterpenoid CDDO-imidazolide (CDDO-Im) not only reduced the lethal effects of LPS (71% survival) but also decreased the infiltration of CD45+ cells into the pancreas and the percentage of Gr1+ myeloid-derived suppressor cell in the spleen of KC mice 4–8 weeks after the initial LPS challenge. While the levels of inflammatory cytokine levels were markedly higher in KC mice versus WT mice challenged with LPS, CDDO-Im significantly decreased the production of IL-6, CCL-2, vascular endothelial growth factor and G-CSF in the KC mice. All of these cytokines are prognostic markers in pancreatic cancer or play important roles in the progression of this disease. Disrupting the inflammatory process with drugs such as CDDO-Im might be useful for preventing pancreatic cancer, especially in high-risk populations.

Introduction

Over 48 000 new cases of pancreatic cancer will be diagnosed in the USA in 2015, and this disease is almost uniformly fatal, with 5-year survival rates still below 10% (1). Pancreatic ductal adenocarcinoma (PDAC) is resistant to conventional cytotoxic chemotherapy and radiotherapy, and more than 80% of patients present with unresectable or metastatic disease. Gemcitabine has been the standard palliative therapy for patients with pancreatic cancer for more than a decade (2). In a phase III clinical trial in patients with advanced pancreatic cancer, a combination chemotherapy regimen composed of FOLinic acid, 5-Fluorouracil, IRINotecan and OXaliplatin (FOLFIRINOX) improved survival 4 months longer than gemcitabine (11.1 versus 6.8 months (3)), but this drug regimen is too toxic for most patients. In 2013, the FDA approved nab-paclitaxel (Abraxane) as a less toxic alternative (4), but this new drug is less effective than FOLFIRINOX, and none of these newer therapies extend survival beyond a year. Although prevention may become the most effective strategy for reducing the unacceptable mortality rates for pancreatic cancer, new drugs and approaches are needed for this devastating disease.

The development of relevant genetically engineered mouse models has provided important biological insights into the

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Abbreviations

CDDO-Im	CDDO-imidazolide
CDDO-Me	CDDO-methyl ester
ELISA	enzyme-linked immunosorbent assay
IHC	immunohistochemistry
LPS	lipopolysaccharide
MDSC	myeloid-derived suppressor cell
OA	oleanolic acid
PDAC	pancreatic ductal adenocarcinoma
TAM	tumor-associated macrophage
Tregs	regulatory T cell
VEGF	vascular endothelial growth factor
WT	wildtype

pathogenesis of PDAC (5). Activating mutations in the *Kras* gene are found in over 90% of human pancreatic cancers. The introduction of a *Kras* mutation specifically in the pancreas of KC mice (genotype = LSL-Kras$^{G12D/+}$;Pdx-1-Cre) closely replicates the genetic background, clinical symptoms and full spectrum of histopathology found in human PDAC (6). A significant inflammatory response is also characteristic of human PDAC (7). Infiltrating immune cells can account for up to 50% of the cells in a pancreatic tumor, and both the immune cells and the tumor produce cytokines and chemokines that suppress antitumor immunity and promote tumor development (8). Notably, early infiltration of immune cells, including tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs), has also been observed in KC mice (9). These cells drive tumor progression, so targeting these cells early in the disease process might be useful for altering the pathogenesis of this disease.

Although activation of Kras during embryonic development is sufficient to induce PDAC, acute and chronic pancreatitis accelerates the development of pancreatic cancer in mice with Kras mutations (10–12). Epidemiologic studies have found that patients with chronic pancreatitis have a 12-fold higher risk of developing PDAC (13). Caerulein, a CCK analog, is one of the most widely used inducers of pancreatic injury in animal models. Despite being a useful reagent for inducing acute pancreatitis, caerulein must be injected almost daily for several months (10) to induce chronic pancreatitis. In contrast, when injected i.p. once a week for 4 weeks (11), lipopolysaccharide (LPS) induces chronic inflammation and early pancreatic lesions in mice with an activating Kras mutation in pancreatic acinar cells. LPS is not routinely used to induce pancreatitis (14) in animal models, even though it is widely used to trigger an inflammatory response, including the release of inflammatory cytokines from macrophages, both *in vitro* and *in vivo*. The effects of LPS-induced inflammation on immune cell infiltration and cytokine secretion in KC mice are not known.

Because of the importance of inflammation and immune cell infiltration in carcinogenesis, anti-inflammatory drugs might be useful interventions (15) for pancreatic cancer. Triterpenoids such as oleanolic acid, found in a variety of plants, have weak anti-inflammatory and anticarcinogenic properties (16). 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid-methyl ester (CDDO-Me) and CDDO-imidazolide (CDDO-Im) are synthetic derivatives of oleanolic acid that are 10 000 times more potent at suppressing production of the inflammatory mediator inducible nitric oxide synthase than the parent oleanolic acid molecule. Synthetic triterpenoids also inhibit the production of IL-1β, IL-6, TNFα, vascular endothelial growth factor (VEGF) and CCL2 in both immune and cancer cells (16). In mice challenged with a low dose of LPS, CDDO-Me suppresses the production of proinflammatory cytokines and alters the composition of immune cell populations in the spleen; CDDO-Me also reduces the number of deaths in mice injected with lethal doses of LPS (17). Moreover, CDDO-Me suppresses angiogenesis *in vivo* (18), reduces the infiltration of TAMs into ER-mammary tumors in PyMT mice (19), reprograms TAMs from an M2 tumor promoting phenotype to an M1 tumor inhibiting phenotype (20) and inhibits the immunosuppressive activity of MDSCs in mice and in cancer patients (21). CDDO-Me is currently being tested in a Phase II clinical trial for the amelioration of pulmonary arterial hypertension; RTA408 (22), a second generation triterpenoid, is being evaluated for its ability to reprogram the immune system and attack tumors by inhibiting MDSC activation and activating cytotoxic T cells. We have previously shown that triterpenoids extend survival in the KPC mouse model of pancreatic cancer but did not study their effects on the tumor microenvironment in this model (23). In addition to characterizing the effects of LPS in KC mice, we tested the ability of CDDO-Im to decrease cytokine production and immune cell infiltration in KC mice.

Materials and methods

Mouse models

All animal studies were done in accordance with protocols approved by the Institutional Animal Care and Use Committee at Dartmouth Medical School and Michigan State University. In KC (LSL-Kras$^{G12D/+}$;Pdx-1-Cre) mice, mutant Kras is expressed in the pancreas (6). These mice were generated by breeding male LSL-Kras$^{G12D/+}$;Pdx-1-Cre and female Pdx-1-Cre mice. For genotyping, the Extract-N-Amp tissue PCR kit (Sigma) was used to extract genomic DNA from tail snips (23). At 4 weeks of age, all KC mice were randomized and fed powdered 5002 rodent chow (LabDiet) for the duration of the studies.

For the initial (Table 1) LPS pilot studies, 9-week-old KC mice, WT LSL-Kras$^{G12D/+}$ or Pdx-1-Cre mice or polyoma-middle T (PyMT) mice on the same C57/BL6 background were injected i.p. with saline (vehicle) or LPS (4mg/kg, Sigma O111:B4 lot 084M4118V) once a week for 4 weeks. In protocol 1 (Figure 1), the pancreas and spleen of KC mice were harvested 4 or 8 weeks after the initial LPS injection (13 or 17 weeks of age). The percentage of CD45+ immune cells in the pancreas and the percentage of GR1+ MDSC in the spleen were analyzed by flow cytometry and by immunohistochemistry (IHC). A portion of the pancreas from these initial LPS studies was also sectioned and stained with hematoxylin and eosin (H & E). In a pilot study of five-paired mice per group harvested 12 weeks after the initial LPS injection, the number of PanINs and areas of PDAC from 4 to 8 sections per mouse were counted. Basal differences in CD45+ and Gr1+ MDSC cells in WT and KC mice were also analyzed by flow cytometry (Supplementary Figure 1, available at *Carcinogenesis* Online). To study acute cytokine production (protocol 2, Figure 2 and Supplementary Tables 1 and 2, available at *Carcinogenesis* Online), 9-week-old KC mice were fed CDDO-Im (24,25) mixed into powdered LabDiet 5002 chow (100mg/kg diet or ~25mg/kg body weight) or control 5002 chow. These diets were started 2 days prior to being challenged with a single dose of LPS (4mg/kg) or saline (vehicle). Twenty-four hours after the injections, plasma and pancreas were harvested and analyzed by a multiplex

Table 1. KC pancreatic cancer mice are susceptible to LPS

Mouse strain	Survivors	P value versus KC mice
LSL-Kras$^{G12D/+}$;Pdx-1-Cre (KC) mice	20/42 (48%)	N/A
Polyoma-middle T (PyMT) mice	16/20 (80%)	0.027
C57BL/6 WT mice	12/12 (100%)	0.0007

Various strains of mice were injected i.p. with LPS (4mg/kg) once a week for 4 weeks. The percentage of survivors one week after the final LPS injection is shown. WT, wildype.

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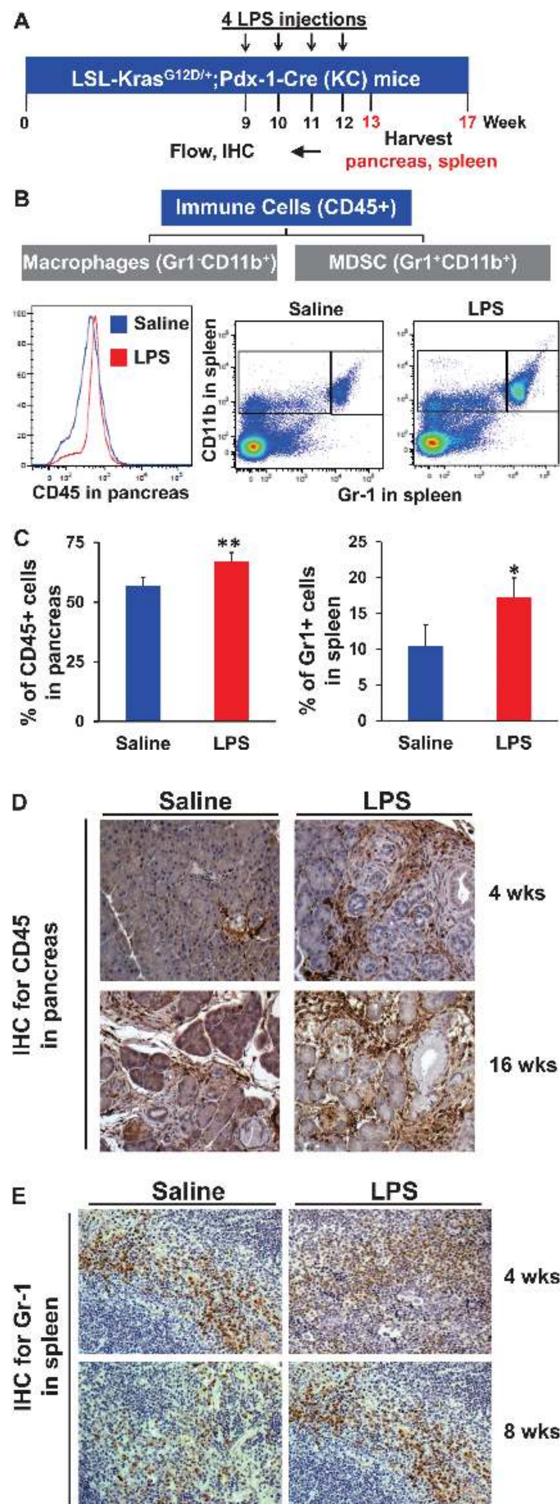

Figure 1. LPS increases the infiltration of CD45+ cells into the pancreas and Gr1+ myeloid derived suppressor cells (MDSC) into the spleen of Kras^{G12D};Pdx1-Cre (KC) mice. (**A**) Nine-week-old KC mice were injected i.p. with saline (vehicle) or LPS (4 mg/kg) once a week for 4 weeks. Four or 8 weeks after the initial LPS challenge, the pancreas and spleen were harvested and analyzed. The % of CD45+ immune cells and the % of CD45+ macrophages (CD11b+GR1–) and MDSCs (CD11b+GR1+) in the pancreas and spleen were analyzed by flow cytometry (**B**, **C**) and by immunohistochemistry (IHC in **D**, **E**). Representative histograms are shown in B. In (C), **P = 0.057 versus saline for CD45+ cells in the pancreas and *P = 0.036 versus saline for GR1+ cells in the spleen; n = 9 paired mice per group. Magnification = 200× in (D) and (E).

cytokine assay or by enzyme-linked immunosorbent assays (ELISAs). In protocol 3 (Figures 3–5 and Supplementary Table 3, available at *Carcinogenesis* Online), 9-week-old KC mice were fed CDDO-Im or control chow. Two days later, mice were injected i.p. with LPS once a week for 4 weeks. Four or eight weeks after the initial LPS challenge, pancreas, spleen and plasma were harvested and analyzed by flow cytometry, IHC, a multiplex cytokine assay and ELISAs. A separate cohort of 9-week-old KC mice (n = 7–8 mice per group) was fed control diet or CDDO-Im in diet for 4 weeks, and immune cells in the pancreas and spleen were detected by IHC (Figure 3E and F).

Flow cytometry

Half of the pancreas and spleen removed from KC mice were minced and incubated separately in digestion media consisting of collagenase (300 U/ml, Sigma), dispase (1 U/ml, Worthington) and DNAse (2 U/ml, Calbiochem) for 30 min at 37°C with stirring. Cells were then passed through a 40-μm Cell Strainer (BD Falcon), and RBC eliminated with lysing solution (eBioscience). Single cells were resuspended in a solution of phosphate-buffered saline/0.5% bovine serum albumin/0.1% azide and stained 1 h at 4°C with the following antibodies: CD45-VioGreen, Gr-1-PE, CD11b-FITC (all Miltenyi, 3 μg/ml) and 5 μg/ml antimouse CD16/CD32 antibody (clone 93, eBioscience) to reduce antibody binding to Fc receptors. Propidium iodide staining was used to exclude dead cells. Cells were analyzed using an eight-color MACSQuant VYB (MiltenyiBiotec) with three laser sources (405, 488 and 561 nm) and FlowJo x.10.0.7r2 software (Tree Star).

IHC

The other half of the pancreas and spleen removed from KC mice were fixed in 10% phosphate-buffered formalin for at least 48 h, embedded in paraffin blocks and sectioned (5–6 μm). Hydrogen peroxide was used to quench endogenous peroxidase activity. Sections were immunostained with CD45 (1:100 eBiosciences) and biotinylated antirat secondary (Vector) or biotinylated Gr1 (1:50 R&D) antibodies. Signal was detected using a Vectastain ABC kit and DAB substrate (Vector) following the manufacturer's recommendations. Sections were counterstained with hematoxylin (Vector). To quantify the % of immune cells in the pancreas, the number of reactive ducts and PanINs containing CD45+ cells was counted in sections from six mice per group in a blinded fashion by two operators. In the spleen, slides were randomized and coded before the % of Gr1+ cells was calculated on four sections per slide from four mice per group.

Multiplex cytokine assay and ELISAs

Plasma from KC mice was aliquoted and stored at –80°C until use. Cytokine levels in plasma were measured using a Millipore mouse 32plex kit (EMD Millipore). Calibration curves from recombinant cytokine standards were prepared with 3-fold dilution steps. Standards and spiked controls were measured in triplicate, samples were measured once and blank values were subtracted from all readings. Assays were carried out in a 96-well filtration plate (Millipore) at room temperature, following the manufacturer's protocol. The fluorescence intensity of the Luminex beads was measured using a Bio-Plex array reader, and Bio-Plex Manager software with five-parametric curve fitting was used for data analysis. Levels of specific cytokines in plasma and pancreas extract were also analyzed by ELISAs (R&D Systems), according to the manufacturer's protocol.

Statistical analysis

Results are expressed as the mean ± SE and were analyzed by t-test or a Mann–Whitney Rank Sum test if the data did not fit a normal distribution. For experiments with more than two groups, data was analyzed by one-way ANOVA followed by a Tukey test, or one-way ANOVA on ranks and Dunn's test (SigmaStat 3.5). Categorical data was analyzed by the Fisher exact test. $P < 0.05$ was considered statistically significant.

Results

KC mice are more sensitive to LPS than WT mice

Daniluk *et al.* (11) recently reported that inflammatory stimuli form a positive feedback loop with oncogenic Ras, prolonging Ras signaling and thus accelerating carcinogenesis in the pancreas. Because infections can cause chronic inflammation, a

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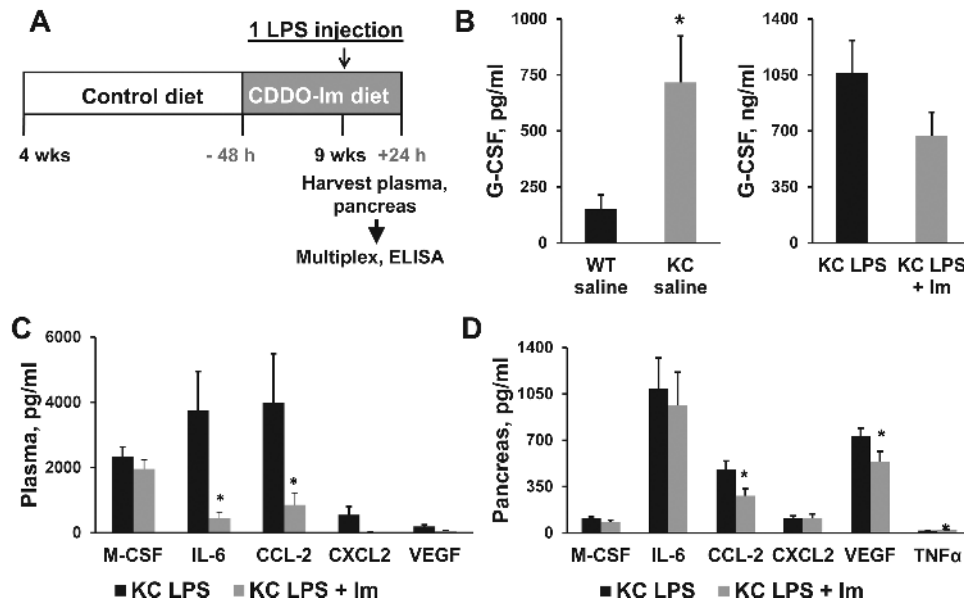

Figure 2. CDDO-Im decreases acute inflammatory cytokine production in mice challenged with LPS. (**A**) Nine-week-old KC (LSL-Kras$^{G12D/+}$;Pdx-1-Cre) or WT (wildtype LSL-Kras$^{G12D/+}$ or Pdx-1-Cre) mice were fed CDDO-Im (100mg/kg diet) or control diet 2 days prior to being challenged with LPS. Mice were then injected i.p. with a single dose of saline (vehicle) or LPS (4mg/kg). Twenty-four hours later, plasma (**B**, **C**) and pancreas (**D**) were harvested and analyzed with a multiplex cytokine assay (Supplementary data, available at *Carcinogenesis* Online) or by ELISAs (B–D). *n* = 8–12 mice per group. In (B) *P = 0.021 WT versus KC mice and *P < 0.05 KC LPS versus KC LPS + Im in (C) and (D).

major risk factor for pancreatitis and pancreatic cancer, they injected mice with 10mg/kg LPS, once a week for 4 weeks. This experimental paradigm increased early PanIN lesions in mice with an activating Kras mutation in the acinar cells of the pancreas but had no effect in WT mice (11). In KC mice, Kras mutations are targeted to the pancreas by the Pdx1-Cre promoter. The Pdx1 transcription factor is expressed in all pancreatic precursor cells during development, but its expression is limited to islet cells in the adult mouse (6). When we used the same protocol of LPS injections in KC mice, this dose was unexpectedly toxic, as 83% (10/12) of the KC mice died within 2 weeks of the initial LPS injection and the majority of these mice died within 48h of the LPS challenge (data not shown). When the LPS dose was reduced to 4mg/kg (Table 1), only 48% (20/42) KC mice survived the LPS challenge. Notably, 100% (12/12) of WT mice on the same genetic background (C57BL/6) as the KC mice survived the lower dose of LPS ($P < 0.05$ versus KC mice). In PyMT mice, also on a C57BL/6 background, expression of the polyomavirus middle T-antigen (*PyMT*) gene is regulated by the MMTV promoter. These mice are widely used to study the role of the immune system in cancer and demonstrate the importance of tumor associated macrophages in the progression of breast cancer. Interestingly, 80% (16/20) PyMT mice lived after the LPS injections, suggesting the inflammation that accompanies tumor development in this strain makes them more susceptible to LPS than WT mice but not as susceptible as the KC mice.

LPS increases the percentage of CD45+ immune cells in the pancreas and Gr1+ MDSCs in the spleen of KC mice

Although tumor immunity may be undermined from initiation in pancreatic cancer (9), we hypothesized that the inflammatory response to LPS would accelerate the infiltration of immune cells. To characterize the immune response, 9-week-old KC mice were injected with 4mg/kg LPS, once a week for four consecutive weeks (Figure 1A). Four or eight weeks after the final injection of LPS, the pancreas and spleen were harvested. Half of each tissue was used for flow cytometry (Figure 1B) and the other half for IHC. CD45, also known as the leukocyte common antigen, is expressed on all leukocytes. Despite inherent variability *in vivo*, the percentage of CD45+ cells detected by flow cytometry was markedly higher ($P = 0.057$) in the pancreas of mice injected with LPS compared to mice injected with saline for nine pairs of littermate-matched mice (Figure 1C). If additional matched pairs of KC mice were included (16–20 weeks after the initial LPS injections), 52.4±3.2% of the cells in the pancreas were CD45+ in the saline group versus 62.3±4% ($P = 0.027$) in mice challenged with LPS (data not shown). The percentage of CD45 + CD11b + Gr1+ MDSC was also significantly ($P < 0.05$) higher in the spleen of LPS-treated mice; MDSCs are immunosuppressive and block the antitumor activity of T cells in PDAC (26,27). In the mice injected with LPS, 17.6±2% of the CD45+ cells in the spleen were Gr1+ compared to only 9.8±2.2% of the cells in mice injected with saline. No changes were detected in the % of CD45+ cells in the spleen, Gr1+ MDSC in the pancreas, or CD45 + CD11b + Gr1− macrophages in the pancreas or spleen (data not shown). To confirm these results, tissue were also analyzed by IHC. In the pancreas, CD45+ staining was higher in sections from the LPS-treated mice than in the saline group (Figure 1D). Notably, CD45+ immune cells were concentrated around early PanIN lesions, which increased in number and severity over time. In the spleen (Figure 1E), Gr1+ MDSC were found in red pulp, not in the T lymphocytes found in white pulp and Gr1+ staining was higher in the groups treated with LPS.

Notably, the immune cell population in the pancreas and spleen of KC mice is different than in WT mice, even without LPS stimulation. As shown in Supplementary Figure 1, available at *Carcinogenesis* Online, there is a significantly ($P < 0.05$) higher percentage of both CD45+ cells in the pancreas and Gr1+ MDSC in the spleen of KC mice than in WT mice. However, LPS can enhance the infiltration of CD45+ and Gr1+ cells in KC mice (Figure 1C–E). To confirm that LPS also accelerated the

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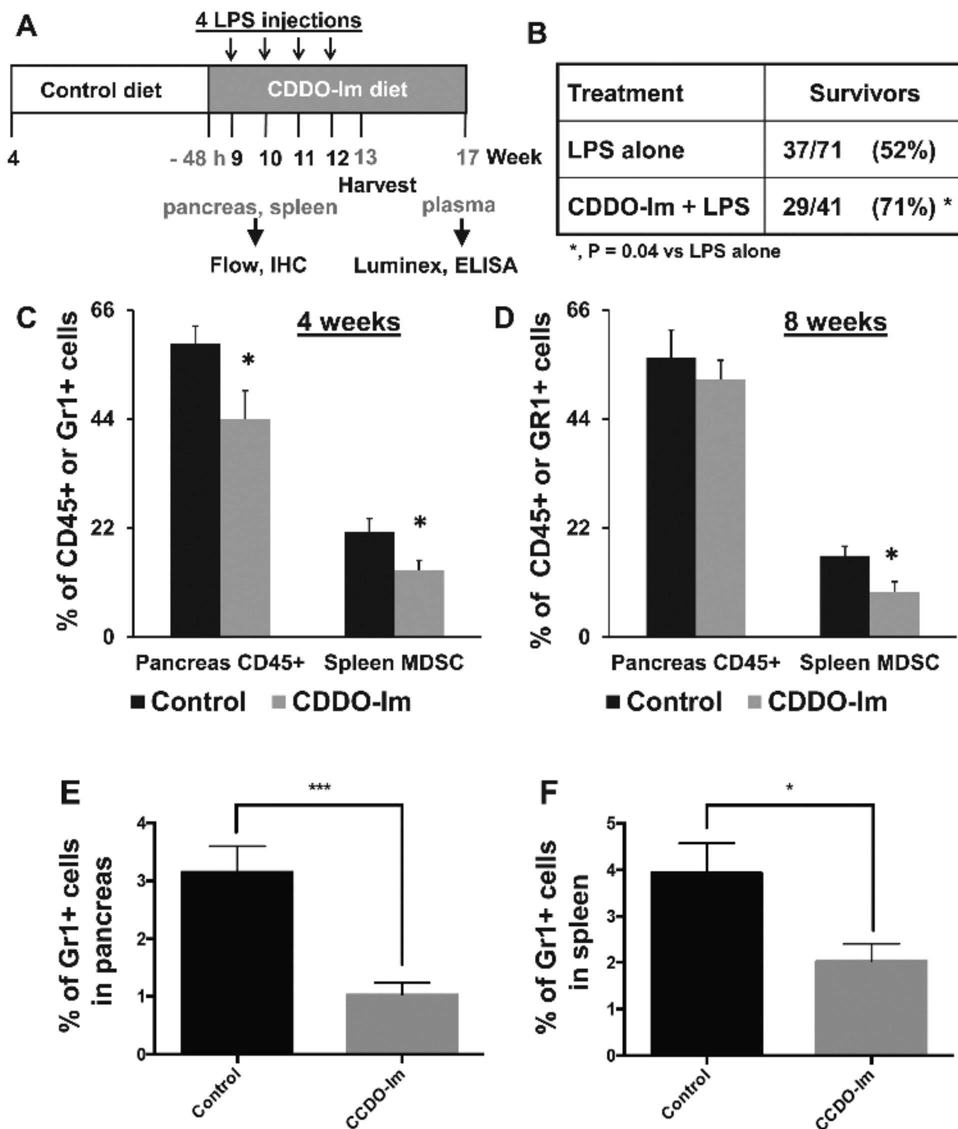

Figure 3. CDDO-Im protects against toxicity induced by LPS and decreases the infiltration of CD45+ immune cells into the pancreas and Gr1+ MDSC into the spleen of KC mice. (**A**) Nine-week-old KC mice were fed CDDO-Im (100 mg/kg diet) 2 days prior to an LPS challenge. Mice were then injected i.p. with saline (vehicle) or LPS (4 mg/kg) once a week for 4 weeks. (**B**) The percentage of survivors 1 week after the final LPS injection. Four (**C**) or eight (**D**) weeks after the initial LPS challenge, the pancreas and spleen were harvested and analyzed by flow cytometry. In (**E**, **F**), KC mice were fed CDDO-Im or control diet for 4 weeks but not challenged with LPS. Pancreas and spleen were harvested 4 weeks later to evaluate immune cell populations. *n* = 8–10 pairs per group; *P < 0.05 versus control; ***P < 0.001 versus control.

development of PDAC in KC mice, the number of pancreatic lesions was evaluated on H & E stained slides. Changes in CD45+ and MDSC populations were observed 4 or 8 weeks after the LPS challenge (Figure 1), but there was not sufficient pathology at these time points to detect differences between groups. At 12 weeks, however, there was a trend toward higher numbers of PanINs in the LPS group (49.4 ± 8.1 per pancreas) than in the saline group (41.8 ± 13.1 per pancreas). The number of PDACs per section was significantly (*P* = 0.03) higher in the pancreas of mice treated with LPS (3.14 ± 0.41) than with saline (1.98 ± 0.15; *n* = 5 mice per group).

Cytokine profiles differ in KC mice versus WT mice challenged with LPS

Because of the importance of inflammation in the pathogenesis of pancreatic cancer (28,29), we compared the cytokine profiles of KC and WT mice 24 h after a single injection of either LPS or saline (Figure 2A). Plasma from 6 to 7 mice per group was analyzed with a multiplex assay (Supplementary Table 1, available at *Carcinogenesis* Online), which is useful for measuring multiple cytokines in a single sample. The only difference between WT and KC mice injected with saline was a markedly higher level of G-CSF, or CSF3, in the KC mice (2116 ± 498 pg/ml) versus WT mice (828 ± 76 pg/ml; *P* = 0.052), suggesting that an activating mutation in Kras mutation is sufficient to increase G-CSF production. Because G-CSF mediates the expansion and recruitment of MDSCs and enhances angiogenesis and metastases (30), high expression of G-CSF is an indicator of poor prognosis in cancer patients. An ELISA was used to confirm these results, and as shown in Figure 2B, endogenous G-CSF levels were more than 4-fold higher in the KC mice than in WT mice (*P* = 0.021; *n* = 8 per group).

LPS increased the secretion of 21 cytokines in WT mice and 29 cytokines in KC mice. Notably, cytokine levels were clearly higher in the KC mice than in the WT mice challenged with LPS for almost all of these cytokines. Because of variability between

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mice, not all of these changes were statistically significant, but the trend is striking. When sample sizes in each group were increased and samples analyzed by ELISAs, LPS significantly ($P < 0.05$) increased levels of G-CSF, IL-6 and VEGF in the plasma (Supplementary Table 2A, available at *Carcinogenesis* Online) and M-CSF, G-CSF, IL-1β, IL-6, CCL-2, CXCL2 and VEGF in the pancreas (Supplementary Table 2B, available at *Carcinogenesis* Online). Many of these cytokines play essential roles in PDAC. IL-6 is emerging as an important target in PDAC, as IL-6 levels are elevated in the serum of patients with pancreatic cancer, which correlates with poor survival (31). Pancreatitis increases IL-6 expression, and the combination of IL-6 and Kras activation enhances MAPK signaling and drives the progression of experimental pancreatic cancer (32). High levels of IL-1β activate NF-κB, resulting in chemoresistance in pancreatic cancer cells (33), and increased IL-1β production as a consequence of a genetic polymorphism reduces survival in pancreatic cancer patients (34). CCL2, also known as MCP-1, mRNA levels are elevated in pancreatic tumors, and this cytokine recruits immunosuppressive macrophages that predict patient survival (35). VEGF can promote tumor angiogenesis and metastasis, and its expression is predictive of recurrence and poor prognosis following resection of PDAC (36). In GEM models of pancreatic cancer, inhibiting the receptor for CSF1 reprogrammed TAMs and improved the efficacy of immunotherapy with PD1 antagonists (37), and the deletion of M-CSF/CSF-1 reduced macrophage infiltration and the burden of pancreatic neuroendocrine tumors (38).

CDDO-Im reduces acute cytokine production in KC mice injected with LPS

The Nrf2/ARE pathway helps regulate the innate immune response to LPS (39), and triterpenoids protect against this inflammatory response by activating the Nrf2 cytoprotective pathway (40). In order to induce Nrf2 target genes, the triterpenoid CDDO-Im (100 mg/kg diet) was fed to KC or WT mice 2 days prior to being challenged with LPS (Figure 2A). This protocol increased mRNA production of the Nrf2 target gene *NQO1* 2-fold in the liver and lungs of these mice (data not shown). Treatment with CDDO-Im also decreased a number of cytokines in KC mice challenged with LPS (Supplementary Table 1, available at *Carcinogenesis* Online), including IFNγ, IL1α, IL-6, IL-7, IL-12 and IL-15, but there were notable differences between KC and WT mice. In WT fed CDDO-Im, levels of IL-1β, IL-9, TNFα and CXCL9 (MIG) were distinctly higher in the LPS + Im group than in the LPS group. In contrast, levels of these cytokines did not change in the KC mice fed CDDO-Im and challenged with LPS. As analyzed by ELISAs, treatment with CDDO-Im significantly ($P < 0.05$) decreased secretion of IL-6 and CCL2 in the plasma (Figure 2C and Supplementary Table 2A, available at *Carcinogenesis* Online). CCL2 and VEGF levels were also significantly ($P < 0.05$) lower in the pancreas of KC mice fed CDDO-Im and challenged with LPS (Figure 2D and Supplementary Table 2B, available at *Carcinogenesis* Online), while levels of TNFα were slightly elevated ($P < 0.05$) in the pancreas of mice in this group. Many of these changes are similar to the cytokine profile reported when CDDO-Im nanoparticles were used to enhance immune surveillance when combined with a HER2 vaccine in a mouse model of breast cancer (41).

CDDO-Im reduces the toxicity of LPS in KC mice

To test the effects of CDDO-Im on chronic cytokine production and immune cell infiltration, KC mice were started on CDDO-Im diet 2 days prior to being challenged with LPS. Mice were injected with four total injections of LPS, once per week and tissues were harvested 4 or 8 weeks after the initial LPS injection (Figure 3A). Although half of the KC mice did not survive this LPS challenge (Table 1 and Figure 3B), 71% (29/41; $P = 0.04$ versus LPS alone) of the mice in the CDDO-Im group survived this protocol. More mice were enrolled in the LPS group in order to generate adequate numbers for analysis, and littermate matched pairs were used when possible.

CDDO-Im reduces the infiltration of immune cells and cytokine production in KC mice challenged with LPS

CDDO-Im significantly ($P < 0.05$) decreased the percentage of CD45+ cells in the pancreas and the percentage of Gr1+ MDSC in the spleen 4 weeks after the initial LPS challenge (Figure 3C), as determined by flow cytometry (Supplementary Figure 2). In the pancreas, 59.3 ± 3.5% of the cells were CD45+ in the control group versus 44 ± 5.6% in the CDDO-Im group ($P = 0.037$). In the spleen, 21.3 ± 2.7% of the cells were Gr1+ in control group versus only 13.5 ± 1.9% in the CDDO-Im group ($P = 0.03$). At 8 weeks after the initial LPS challenge (Figure 3D), there was no difference in the percentage of CD45+ immune cells in the pancreas between the 2 groups. However, the percentage of Gr1+ MDSC in the spleen was still significantly ($P = 0.02$) lower in the KC mice fed CDDO-Im than in the LPS control mice at this time point. In a separate cohort of KC mice fed control diet or CDDO-Im in diet for 4 weeks but not challenged with LPS, there were no differences in the percentage of CD45+ cells in the pancreas of the mice (data not shown). As shown in Figure 3E and F, the percentage of Gr1+ MDSC was significantly ($P < 0.05$) lower in both the pancreas and spleens of KC mice fed CDDO-Im versus control diet. In these mice, the levels of G-CSF in plasma were markedly lower in the CDDO-Im group (373.5 ± 80.5 pg/ml) than in the control group (662.9 ± 120.2 pg/ml; $P = 0.0614$).

In order to confirm the flow cytometry data, tissues were also analyzed by IHC. As shown in Figure 4A, abundant CD45+ immune cells surround reactive ducts and PanINs in the pancreas. In the slides from mice treated with CDDO-Im, 60.5 ± 9.2% of the normal or reactive ducts were surrounded by CD45+ cells versus 73.8 ± 4.6% ($P = 0.086$) in slides from the LPS control group. In PanIN lesions, the cells de-differentiate from a cuboidal to columnar shape, display abundant cytoplasm and nuclear atypia, produce mucus and lose polarity (6). In the LPS group, 86.5 ± 6.5% of the PanIN lesions are surrounded by CD45+ cells compared to only 55 ± 11% ($P = 0.012$) of the PanINs in the CDDO-Im group (Figure 4B). In the spleen (Figure 4C), Gr1+ MDSC surrounded the T cells in the white pulp. CDDO-Im reduced the percentage of these cells by half, as 21.5 ± 2.3% of the cells were Gr1+ in the LPS group versus only 9.2 ± 1.4% ($P < 0.001$) of cells in sections from mice treated with CDDO-Im (Figure 4D).

To evaluate chronic changes in the cytokine profile, plasma from KC mice 4 and 8 weeks after the first LPS challenge was analyzed using a multiplex assay (Figure 5A and B and Supplementary Table 3, available at *Carcinogenesis* Online) or ELISAs (Figure 5C and D). The pancreas in these studies was used for flow cytometry and IHC and thus was not available to compare to the acute changes in cytokine levels detected in Figure 2D. Chronic cytokine levels were much lower at these time points than at 24 h after injection (Figure 2 and Supplementary Table 1, available at *Carcinogenesis* Online). Notably, levels of the chemoattractants eotaxin and MIG/CXCL9 were significantly ($P < 0.05$) lower in the plasma of mice treated with CDDO-Im 4 weeks after the initial LPS injection (Figure 5A). Plasma levels of eotaxin are elevated in patients with metastatic pancreatic cancer (42), and inhibition of NF-κB and its targets, including eotaxin, prolonged

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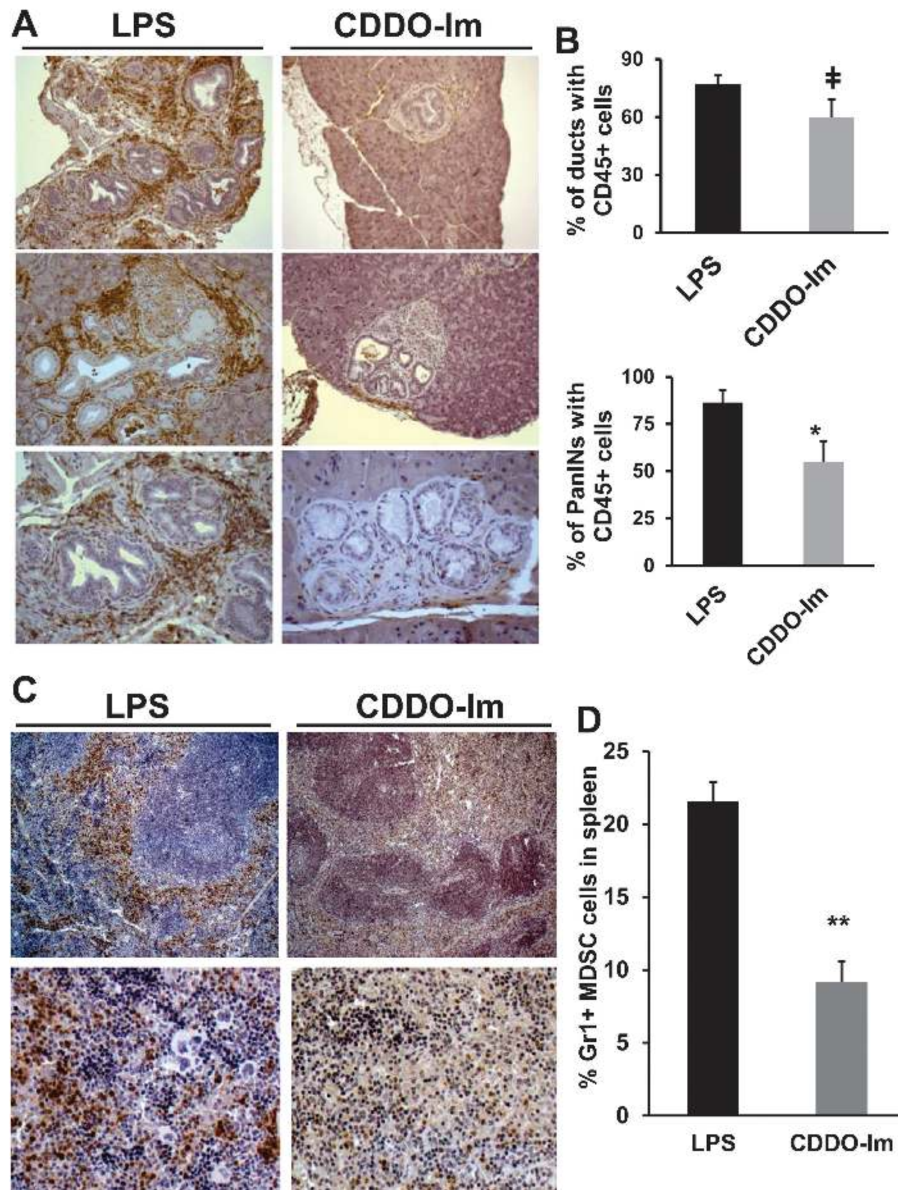

Figure 4. CDDO-Im reduces CD45+ cells in the pancreas and Gr1+ cells in the spleen, as confirmed by IHC. Nine week old KC mice were fed CDDO-Im (100 mg/kg diet) 2 days prior to being challenged with LPS. Mice were then injected i.p. with saline (vehicle) or LPS (4 mg/kg) once a week for 4 weeks. One week after the final LPS injections, the pancreas (**A**, **B**) and spleen (C,D) were harvested and analyzed by IHC. Samples were randomized and blinded, and the % of CD45+ cells (brown) in the entire pancreas (B, upper panel) or in pancreatic intraepithelial neoplasias (PanINs; B, lower panel) were analyzed. In the spleen (**C**, **D**), the % of Gr1+ MDSC (brown) were calculated. Magnification in A = 200× upper four panels and 400× lower two panels and in C = 100× upper two panels and 200× lower two panels. *n* = 4–6 mice per group; ‡P = 0.086; *P < 0.05; **P < 0.001.

survival in a GEM model of experimental PDAC (43). IL-5 was decreased in the CDDO-Im group, and G-CSF (Figure 5B) levels were noticeably lower in mice treated with CDDO-Im versus the control group (*P* = 0.088). IL-6 levels (Figure 5C) were also lower following CDDO-Im treatment, especially at 4 weeks (12.9 ± 5.2 pg/ml versus 3.7 ± 0.6; *P* = 0.077). When G-CSF levels were measured by ELISA (Figure 5D), 928 ± 261 pg/ml were detected in the control group versus 413 ± 71 pg/ml in the CDDO-Im group (*P* = 0.026), a reduction of more than 50%.

Discussion

These studies are the first to characterize the effects of LPS on immune cell infiltration and cytokine secretion in KC mice and to test whether the potent anti-inflammatory triterpenoid CDDO-Im could impede these changes. KC mice were more sensitive to LPS than other strains, and repeated doses of LPS increased the percentage of CD45+ immune cells in the pancreas and the number of Gr1+ MDSCs in the spleen 4–8 weeks after the original inflammatory stimulus. Cytokine secretion was also clearly higher in the KC mice than in WT mice challenged with LPS for a number of cytokines known to contribute to the progression of PDAC, including G-CSF, M-CSF, IL-6, CCL2 and VEGF. CDDO-Im not only decreased production of IL-6, CCL2, G-CSF and VEGF but also reduced the infiltration of CD45+ immune cells into the pancreas and MDSCs into the spleen. Because an inflammatory environment is required for the pathogenesis of pancreatic cancer driven by constitutive Kras activation, these results suggest that anti-inflammatory drugs such as CDDO-Im should be considered for delaying or preventing this disease.

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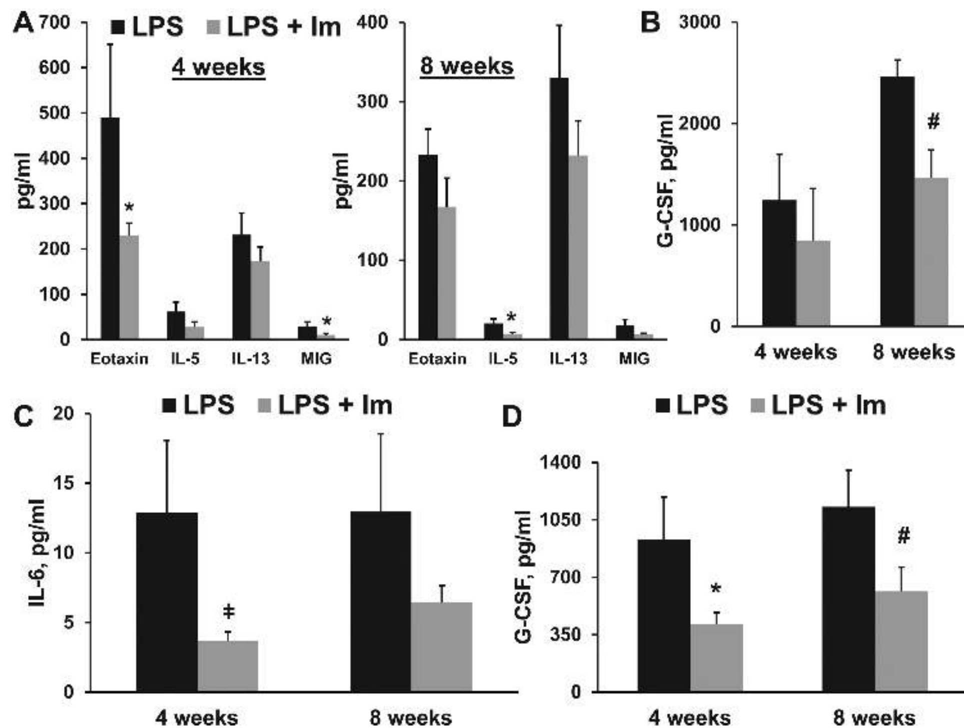

Figure 5. CDDO-Im reduces cytokine levels in plasma in KC mice challenged with LPS. Nine week old KC mice were fed CDDO-Im (100mg/kg diet) 2 days prior to an LPS challenge. Mice were then injected i.p. with saline (vehicle) or LPS (4mg/kg) once a week for 4 weeks. Four or eight weeks after the initial LPS challenge, plasma was harvested and analyzed by a multiplex cytokine assay (**A**, **B**) or ELISA (**C**, **D**). In the multiplex assay in (A) and (B), *n* = 12 pairs at 4 weeks and *n* = 8 pairs at 8 weeks; *P < 0.05 versus LPS control; #P = 0.08. In (C and D), *n* = 8–12 pairs; ‡P = 0.077; *P < 0.05; #P = 0.06.

Although a number of adults harbor Kras mutations in a variety of tissues (44), these mutations must be accompanied by chronic inflammation in order to drive tumor progression (10). Pancreatitis is a known risk factor for PDAC, and bacterial infections may also contribute to the development of this disease (45). In our studies, KC mice, with Kras mutations directed to the pancreas, were highly sensitive to LPS from the cell wall of Gram-negative bacteria, as shown by the increased lethality and higher cytokine levels in plasma than in the WT mice (Table 1 and Supplementary Table 1, available at *Carcinogenesis* Online). Although there is no simple explanation for the increase in lethality from LPS in KC mice, elevated TNFα levels are known to drive lethality from septic shock (46). In our studies, however, death is likely the result of a cytokine storm, and not a single cytokine, as levels of more than 25 different cytokine were markedly higher in the KC mice than in the WT mice challenged with LPS.

Caerulein, a CCK analogue derived from an Australian tree frog that induces secretion of pancreatic enzymes, is a well-characterized inducer of exocrine insufficiency in the pancreas (14). Despite being an excellent reagent for inducing acute pancreatitis, caerulein is expensive and requires repetitive dosing over prolonged periods [up to five injections per week for 6–10 months (10)] to induce the chronic pancreatitis that is most relevant to human PDA. In contrast, LPS can induce chronic inflammation and accelerate the development of PDAC in mice with Kras mutations with just four injections [Figure 1 and (11)]. Despite the known systemic effects of LPS, it also has pancreas-specific effects, as shown by the elevation in M-CSF, G-CSF, IL-7, CCL2, CXCL2, VEGF and IL-1β in the pancreas of KC mice challenged with LPS (Figure 2 and Supplementary Table 2B, available at *Carcinogenesis* Online). Taken together, these results suggest that the use of LPS in KC mice can be useful for studying the role of inflammation and immune cell infiltration in the development of PDAC as well as for testing new drugs.

Immunoprevention and immunotherapy are among the most exciting new areas of cancer research (8,47). Immune cells contribute to the inflammatory process that accelerates PDAC (7,29). While LPS increases the percentage of CD45+ cells in the pancreas and Gr1+ cells in the spleen of KC mice (Figure 1), CDDO-Im decreases the infiltration of these cells, as confirmed by both flow cytometry and IHC (Figures 3 and 4). The effects of this drug on MDSC are important, as these immature, immunosuppressive cells block a cytotoxic T cell response (7). A number of drugs that target these cells are being tested in clinical trials (48), including triterpenoids [(21) and NCT02259231]. In contrast to a previous study by Nagaraj *et al.* in which CDDO-Me inhibited the functional activity of MDSC without altering the number of these cells (21), we found that CDDO-Im decreased the percentage of MDSC in the spleens of KC mice (Figures 3 and 4). In addition to different triterpenoids being used for the two studies, subcutaneous tumor models were used and mice were only treated for 7 days in Nagaraj's experiments. In our studies, KC mice develop autochthonous pancreatic tumors and mice were treated with drugs for 4–8 weeks, and this longer time period may be needed to reduce the numbers of MDSC in tissues.

Notably, CDDO-Im also reduces inflammatory cytokines and growth factors highly relevant to pancreatic cancer. In the experiments following an acute challenge with LPS (Figure 2), CDDO-Im suppressed levels of CCL2, which recruits monocytes to sites of inflammation, and VEGF, which promotes angiogenesis, in the pancreas. These findings mirror results we have obtained with the triterpenoid CDDO-Me; we have recently shown that CDDO-Me markedly attenuates CCL2 and VEGF expression in PyMT tumor associated macrophages both *in vitro* and *in vivo* (20). Elevated levels of both CCL2 (35), and VEGF (36) are

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poor prognostic indicators in patients with PDAC. Furthermore, CDDO-Im decreased IL-6 and G-CSF 4–8 weeks after chronic LPS exposure (Figure 5). IL-6 is a pro-inflammatory cytokine with pleiotropic effects; serum levels of IL-6 are frequently elevated in pancreatic cancer patients at advanced stages and contribute to cachexia and poor survival (31). High levels of G-CSF in patients with PDAC correlate with poor survival (30). Because this cytokine regulates the mobilization of $Cd11b^+;Gr1^+$ MDSC, the reduction in MDSC in KC mice treated with CDDO-Im is consistent with the inhibition of G-CSF observed in Figures 4-5. Exclusion of myeloid-derived cells, including MDSCs and macrophages, from the pancreatic tumor microenvironment has potential therapeutic value in PDAC, as these cells have been shown to foster angiogenesis, metastasis, and immune suppression (49). In this regard, our results suggest that CDDO-Im may enhance immune activation in PDAC by inhibiting immune-suppressive MDSC infiltration and by enhancing expression of T cell-activating cytokines, including IL-1β and TNFα (Supplementary Table 1, available at *Carcinogenesis* Online). Intriguingly, we have shown that CDDO-Me redirects activation of breast tumor associated macrophages from an M2 immune-suppressive to M1 immune-stimulatory phenotype (20). Although beyond the scope of the present study, our future work will determine whether CDDO-Im mediates similar effects on PDAC tumor associated macrophages.

Characterizing the effects of LPS on KC mice and establishing the inhibitory properties of CDDO-Im on immune cell infiltration and cytokine secretion in KC mice are important results. Future studies will test whether CDDO-Im or other drugs that suppress inflammation or pancreatitis can also delay the progression of pancreatic cancer. Because LPS accelerates carcinogenesis in KC mice (Figure 1), there should be sufficient pathology to detect differences in drug treatment 16–20 weeks after the LPS challenge. This time frame is significantly shorter than the 38–50 weeks (50,51) used by other groups to detect meaningful differences between treatment groups in KC mice. CDDO-Im and CDDO-Me are comparable in efficacy in almost all *in vitro* assays, although there are some differences in the subset of genes regulated by these triterpenoids (52). CDDO-Im was used in these studies as a proof of principle to demonstrate that a triterpenoid with an imidazolide ring is active *in vivo* and should be considered for further development. The ability of CDDO-Im to inhibit tumor progression as well as modulate immune cell populations will be addressed in future experiments.

If successful, this model could be adapted for rapid drug screening *in vivo*, with early effects on cytokines or immune cell infiltration as potential biomarkers. Chemoprevention of cancer is a realistic, practical approach to control cancer, especially in high risk patient populations, as has been shown in animal models of cancer at numerous sites and in the clinic for head and neck, breast and colon cancer (53). In addition to patients with pancreatitis, at least two other subpopulations of patients would be excellent candidates for safe and effective drugs to prevent pancreatic cancer. Up to 10% of pancreatic cancers are familial, and the lifetime risk of developing pancreatic cancer in individuals who have at least three first-degree relatives with pancreatic cancer is 32 times higher than the general population (54). Some of these high-risk individuals have resorted to prophylactic pancreatectomy to avoid the dismal prognosis of pancreatic cancer. Moreover, the increased use of CT and MRI scans has revealed that approximately 1% of individuals undergoing abdominal scans have asymptomatic pancreatic cysts or 'incidentalomas.' Over half of these cysts are mucinous neoplasms or preneoplastic lesions that may progress to pancreatic cancer, and thousands of patients have now been identified with these lesions. Conservative management and careful monitoring are usually recommended for these asymptomatic cysts, while resection is often performed on the precursor mucinous lesions (55). Anti-inflammatory agents or multifunctional drugs such as triterpenoids that target both immune cells and cancer cells (16) should be considered for patients at high risk for developing PDAC, especially as screening for Kras mutations improves and more people harboring these potentially lethal mutations are identified.

Supplementary material

Supplementary Tables 1–3 and Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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Conflict of Interest Statement: These studies were supported by a research grant from Reata Pharmaceuticals, Inc., and Karen Liby and Michael Sporn have patent interests in synthetic triterpenoids.

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