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Pancreas-specific deletion of protein kinase D attenuates inflammation, necrosis, and severity of acute pancreatitis

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Abstract

Background—Protein kinase D (PKD) family, which includes PKD/PKD1, PKD2, and PKD3, has been increasingly implicated in the regulation of multiple cellular functions and human diseases. We recently reported that pharmacologic inhibition of PKD ameliorated the pathologic responses and severity of pancreatitis. However, to further investigate the importance of PKD family members in pancreatitis, it is necessary to explore the effects of pancreas-specific genetic inhibition of PKD isoform on pathology of pancreatitis.

Methods—We generated a mouse model (referred as PKD3*Δpanc* mice) with pancreas-specific deletion of PKD3, the predominant PKD isoform in mouse pancreatic acinar cells, by crossing *Pkd3flox/flox* mice with *Pdx1-Cre* transgenic mice which express Cre recombinase under the control of the mouse *Pdx1* promoter. Pancreas-specific deletion of the PKD3 gene and PKD3 protein was confirmed by PCR and Western blot analysis. Experimental pancreatitis was induced in PKD3*Δpanc* and *Pkd3flox/flox* (control mice) littermates by intraperitoneal injections of cerulein or L-arginine.

Results—Compared to the control mice, PKD3*Δpanc* mice displayed significant attenuation in inflammation, necrosis, and severity of pancreatitis in both experimental models. PKD3*Δpanc*

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CRediT Author Statement

Jingzhen Yuan: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Supervision, Writing- Reviewing and Editing. **Chintan Chheda**: Methodology, Investigation. **Honit Piplani:** Investigation. **Meng Geng**: Investigation. **Grace Tan**: Investigation. **Reetu Thakur**: Investigation. **Stephen J Pandol:** Conceptualization, Supervision, Reviewing and Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

mice had markedly decreased NF-κB and trypsinogen activation, pancreatic mRNA expression of multiple inflammatory molecules, and the receptor-interacting protein kinase 1 (RIP1) activation in pancreatitis. PKD3*Δpanc* mice also had less pancreatic ATP depletion, increased pro-survival Bcl-2 family protein expression, and autophagy promotion.

Conclusion—With PKD3*Δpanc* mouse model, we further demonstrated that PKD plays a critical role in pathobiological process of pancreatitis and PKD constitutes a novel therapeutic target to treat this disorder.

Keywords

genetic deletion of PKD3; pancreatitis; NF-κB; necrosis; autophagy

1. Introduction

Acute pancreatitis is a serious medical disorder and a leading gastrointestinal cause of hospitalization and mortality in the United States [1–5]. Inflammation, inappropriate intracellular activation of digestive enzymes, and parenchymal acinar cell injury and necrosis are the critical pathophysiologic processes of acute pancreatitis. Although mortality is low in patients with mild forms of acute pancreatitis, the mortality in severe necrotizing pancreatitis is 15% and increases to 35% when associated with persistent organ failure [6,7]. There have been significant advances in evidence-based management of patients with acute pancreatitis [1,3], but the field still lacks therapeutics targeting the molecular pathogenesis of the disease representing a major unmet need in medicine [1,8]. Thus, elucidating the key molecular signals that mediate these pathobiological processes is important for developing new therapeutic strategies.

Increasing evidence has demonstrated that inflammation in pancreatitis is regulated substantially by the pro-inflammatory transcription factors NF-κB. NF-κB activation promotes the expression of pro-inflammatory cytokines, chemokines, immune receptors, and other inflammatory molecules [9–12], which are responsible for both pancreatic inflammation and the severe systemic inflammatory complications of the disease [9,13–16]. Multiple experimental models of pancreatitis have shown that NF-κB activation in acinar cells is one of earliest events in acute pancreatitis and the inhibition of NF-κB activation alleviates the severity of the disease [11,12,16,17]. Further, NF-κB activation within pancreas directly triggered by adenovirus-mediated gene transfer can initialize pancreatic and systemic inflammatory responses [15]. The important role of NF-κB in pancreatitis provides an approach to prevent severe pancreatitis in early stage of the disease by targeting the molecular signals modulating NF-κB activation.

Understanding the regulation of the necrotic death pathways in acute pancreatitis is important because the severity of acute pancreatitis in both humans and in experimental models correlates with the extent of necrosis [18]. A number of reports indicated that the receptor-interacting protein kinase 1 (RIP1) is a key mediator of programmed necrosis in many diseases [19–26, particularly in pancreatitis [23–26]. Degradation or inactivation of RIP1 has been suggested to be one of mechanisms protecting acinar cells from necrosis in cerulein-induced pancreatitis [23,25,26]. Bcl-2 proteins are known important regulators of

mitochondrial permeabilization [27,28]. The recent studies of our group demonstrated that the predominant effect of pro-survival Bcl-2 proteins, Bcl-2 and Bcl-xL, is to stabilize inner membrane integrity and block the mitochondrial permeability transition pore opening, thus maintaining energy production and preventing necrosis [29,30]. Bcl-xL/Bcl-2 inhibition with either pharmacological Bcl-xL/Bcl-2 inhibitors or with specific siRNA in pancreatitis promoted ATP depletion and necrosis but not apoptosis [29,30]. Therefore, the pro-survival Bcl-2 proteins are now recognized to play an important role in protection of acinar cells from necrosis by stabilizing mitochondria against death signals.

Premature trypsinogen activation is an early event in acute pancreatitis [31]. Disorder of autophagy participates in the intracellular trypsin activation in pancreatitis [32]. Autophagy is the process of removal of damaged cellular components during stress conditions through a sequential process of autophagosome-lysosome fusion, formation of autolysosome, and the subsequent digestion of cargo [33]. It has been increasingly recognized that impaired autophagosome-lysosome fusion is a key pathologic response occurring in pancreatitis [34,35]. The disorder in autophagy can contribute to pathologic process of pancreatitis including pancreatic vacuolization, trypsinogen activation, cell death and inflammation [34]. We recently reported that impaired autophagic flux in pancreatitis was due to altered levels of lysosome-associated membrane glycoprotein (LAMP), 5′ AMP-activated protein kinase (AMPK) and Unc-51 like autophagy activating kinase 1 (ULK-1), resulting in autophagosome accumulation (incomplete autophagy) [35]. The study showed that cerulein-induced pancreatitis was associated with inhibition of the activated states of ULK1 and AMP kinase. Treatment of the animals with simvastatin reversed the inhibited ULK1 and AMP kinase to the activated state, thus enhanced autophagic flux and restored autophagy. Consequently, the enhanced/normalized autophagy resulted in inhibition of inappropriate conversion of trypsinogen to trypsin and was associated with decreased the infiltration of inflammatory cells, reduced the accumulation of vacuoles, and necrotic cell death. Thus, enhanced autophagic flux have been determined to play a vital role in the protective effects of simvastatin in attenuating pancreatitis responses pancreatitis [35].

Protein kinase D, a novel serine/threonine protein kinase family, is composed of PKD/PKD1, PKD2, and PKD3. The three isoforms of PKD family have similar overall structure and primary amino acid sequence with 70~85% overall identity at the amino acid level. Particularly, their catalytic domains and regulatory DAG-binding domains, which are critical for controlling the intracellular localization of these protein kinases, are highly homologous. Because of these similarities, the three PKD family members share common regulatory mechanisms in many cell types [reviewed in 36–38]. PKD family members have emerged as key participants in the signal transduction pathways and are increasingly implicated in the regulation of multiple cellular functions and human diseases [36–38].

Of significant importance for pancreatitis, the studies from our group and others have shown that both PKD1, the predominant PKD isoform in rat exocrine pancreas, and PKD3, the major PKD isoform in mouse exocrine pancreas, can be activated by a number of gastrointestinal secretagogues in pancreatic acini [39–41,31]. The secretagogue-stimulated activation and phosphorylation of either PKD1 or PKD3 is predominantly through a PKC-dependent but Ca^{+2}- independent pathway [39–41]. With rat experimental models, we

further demonstrated that PKD/PKD1 activation was required for key pathological features of acute pancreatitis, including NF-κB activation [40], acinar cell necrosis [25], and zymogen activation [31]. PKD3 has been implicated in hormone-stimulated pancreatic exocrine secretion by another research group [41].

We have recently shown that novel small molecule PKD inhibitors CID755673 and CRT0066101 that inhibits all the three isoforms of PKD family attenuated inflammation, zymogen activation, acinar cell necrosis, and the severity of pancreatitis in both *in vitro* and *in vivo* experimental models of the two rodents [31,25,42,43].

Studying the regulatory mechanisms of PKD1 and PKD3 is important for human health since evidence from the human atlas project establishes that PKD1 and PKD3 are both moderately well expressed in human exocrine pancreas. To further define the functions of PKD in pancreatitis and validate PKD as a significant target for pancreatitis, we recently developed a genetic mouse model with pancreas specific deletion of PKD3, the major PKD isoform in mouse pancreatic acinar cells. We designed the present study to explore the mechanisms through which PKD regulates inflammation, necrosis and cell injury in experimental models of acute pancreatitis.

Our findings here showed that pancreas-specific deletion of PKD3 decreased inflammation, necrosis, cell injury and the severity of acute pancreatitis in two experimental mouse models, cerulein-induced pancreatitis and L-arginine- induced pancreatitis. PKD3 mediated inflammation in pancreatitis through NF-κB activation-promoted production of inflammatory molecules. PKD3 promoted necrosis and cell injury in pancreatitis through mechanisms involving mitochondrial pro-survival Bcl-2 family proteins, ATP depletion, decreased RIP1 degradation, cathepsin-dependent pancreatic trypsinogen activation, and impaired autophagy flux. Thus, we suggest that PKD is a potential target for prevention and/or treatment of acute pancreatitis.

2. Methods

2.1. Development of the genetic mouse model with pancreas-specific deletion of PKD3 (referred as PKD3Δpanc mice)

PKD3*Δpanc* mice were developed by crossing *Pkd3flox/flox* mice (referred as PKD3*F/F* mice, from Dr. Doreen Cantrell, University of Dundee Scotland) with *Pdx1-Cre* transgenic mice (from The Jackson Laboratory) which express Cre recombinase under the control of the mouse *Pdx1* promoter. Both strains were C57BL/6J background. PKD3*F/F* strain mice contain a conditional *Pkd3 (Prkcn*) allele that could be deleted specifically in pancreatic cells. LoxP sites were inserted into the *Prkd3* locus to flank exons 6 and 7. Deletion of exons 6 and 7 was achieved by breeding PKD3*F/F* mice to *Pdx1-Cre* mice, resulting in pancreas-specific frameshift conditional knockout mice, namely, PKD3*Δpanc* mice. The resulting strain exhibits specific deletion of PKD3 in pancreatic cells including acinar cells.

Genotyping of PKD3-flox mice was carried out by PCR of genomic DNA using primers 1341_25 (5'GGC-AGT-ACA-CAT-TTT-GAA-GGC3') and 1341_26 (5'ACA-ACA-GAGTGC-CAC-TCA-GC3'), producing products of 279 bp [PKD3WT (wild-type PKD3)]

and 398 bp (PKD3-flox). Genotyping of PKD3*F/FPdx1-Cre* mice was carried out by PCR of genomic DNA using primers recommended by The Jackson Laboratory (on line *Pdx1-Cre* transgenic mice information), producing products of 100 bp (*Pdx1-Cre*) and 324 bp (internal control). PCR of pancreas and liver tissue extract to confirm pancreas-specific Cre deletion of *Pkd3* gene was carried out by using primers 1341_25 (sequence see above), 1341_27 (5'CAC-AAG-TAC-GAAGAG-AGG-AGA-ATC3'), and 1341_29 (5'GGCACTCAGAACTGGAACC3'), producing products of 417 bp [PKD3WT], 553 bp (PKD3-fl), and 267 bp (PKD3-CON KO).

2.2. Animal care guidelines

Breeding of the PKD3*Δpanc* mice and control mice and handling of the animals were approved by the Animal Research Committee of the VA Greater Los Angeles Healthcare System, and Cedars-Sinai Medical Center in accordance with the National Institutes of Health guidelines. The animals were kept in a temperature (23±2°C) and humidity (55% ±5%) controlled room with a 12-h light/dark cycle (lights on at 07:00am). The animals were provided *ad libitum* standard mouse chow and tap water. All experiments were performed according to the guidelines of the National Institutes of Health guidelines.

2.3. Experimental pancreatitis

Acute pancreatitis was induced in PKD3*F/F* mice (as WT control) and PKD3*Δpanc* mice (males, 6–8 weeks old, 25–30g, 8–10 mice in each treatment group) by up to 7 hourly intraperitoneal (IP) injections of 50μg/kg cerulein or by 2 hourly IP injections of 4g/kg L-arginine. Control animals received similar injections of physiologic saline. In the cerulein model, the mice were euthanized by CO_2-induced asphyxiation at 30min and 7h after the first injection. In the arginine model, mice were sacrificed 72h after the 1st injection. The blood and pancreas were harvested for measurements. Tissues were immediately removed, frozen in liquid nitrogen and stored at −80°C.

2.4. Pancreatic tissue lysate preparation and Western blot analysis

Portions of frozen tissue were homogenized on ice in RIPA buffer supplemented with 1 mM PMSF and a protease inhibitor cocktail containing pepstatin, leupeptin, chymostatin, antipain and aprotinin (5μg/ml each). After sonication the lysates were rotated for 40 min and centrifuged at 4 °C for 15 min at 16,000g. The supernatants were collected and stored at −80°C. Western blot analyses were performed as described previously [25,40,42]. The membranes were blocked by 1–2 h incubation with 5% nonfat dried milk in Tris-buffered saline, pH 7.2 and probed overnight at 4 °C with specific primary antibodies at a 1:500–1:1000 dilution in the Tris-buffered saline containing 3% nonfat dried milk. Then the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase at 1:5000 dilutions for 1 hour at room temperature. Blots were developed by using the enhanced chemiluminescence detection kit (Pierce). When re-probing was necessary, the membrane was stripped of bound antibody by incubating in Re-Blot Plus Mild Solution (Millipore, Temecula, CA) for 20 min.

Primary antibodies used were as follows: antibodies against pancreatic lipase, PKD C-20, PKCε, PKCδ, and Bcl-2 (from Santa Cruz Biotechnology, Santa Cruz, CA). α-amylase

antibody (from Sigma-Aldrich); PKD3 antibody (from Cell Signaling, Beverly, MA; and Bethyl Laboratories, Montgomery, TX). PKD2 antibody (from Bethyl Laboratories, Montgomery, TX). RIP1 antibody (from Cell Signaling and BD Science, San Diego, CA). Antibodies against NF-κB P65, IκB-α, Bcl-xL, high-mobility group protein 1 (HMGB1), GAPDH or ERK1/2, LC3-II and p62, pULK1 (S555) and pAMPK (T172) (from Cell Signaling, Beverly, MA).

2.5. Enzymatic assays

Animal serum amylase and lipase activities were determined by Antech Diagnostics (Irvine, CA) Custom Service. Amylase activity in isolated pancreatic acini and the incubation medium was determined by using a commercial kit (Phaebadas kit; Pharmacia Diagnostic, Rochester, NY) as described [31]. Amylase secretion was calculated as the percent total cellular amylase released into the medium [medium / (medium + cells)]. Caspase 3 activities in pancreatic tissue homogenates were measured using a fluorogenic assay with substrates specific for caspase-3 (Ac-DEVDAMC) as we described previously [25,26]. Active trypsin in pancreatic tissue homogenates was measured using Boc-Gln-Ala-Arg-AMC (Bachem Americas Inc, Torrance, CA) as a substrate by a fluorogenic assay as described previously [31,42]. Cathepsin B activities in pancreatic homogenates were measured as described previously [31] using fluorogenic cathepsin B substrate III: Z-Arg-Arg-AMC, 2HCl (Millipore Sigma, Burlington, Massachusetts). ATP level in pancreatic tissue extracts was measured according to the manufacturer's instructions using luciferin/luciferase-based ATP determination kit (#A22066, Thermo Fisher Scientific, Grand Island, NY).

2.6. Preparation and treatments of dispersed pancreatic acini and preparation of cell lysate

Pancreatic acini were isolated from male PKD3*F/F* mice (as WT control) and PKD3*Δpanc* mice using a collagenase digestion method as described previously [25,31,40,29]. For experimental purposes, the acini were incubated in medium 199 and then treated with CCK, as described [25,31,40,29]. Aliquots of the acinar cells were resuspended in the RIPA buffer and processed as described above for tissue samples.

2.7. Quantification of necrosis

Quantification of necrosis in *in vivo* pancreatitis was performed on pancreatic tissue (collected after 7 hourly cerulein injections or from L-arginine pancreatitis model) sections stained with H&E. Cells with swollen cytoplasm, loss of plasma membrane integrity, and leakage of organelles into interstitium were considered necrotic. A total of at least 2000 acinar cells were counted on tissue sections from each animal and 4–5 animals per condition were counted.

Necrosis in pancreatic acini was determined by the release of lactate dehydrogenase (LDH) and high-mobility group protein-1 (HMGB1) into the incubation medium, as described previously [25,29,44]. LDH activity was measured using Cytotoxicity Detection Kit (Roche Diagnostics, Indianapolis, IN). HMGB1 was measured with Western blot analysis for the incubation media and cell homogenates using HMGB1 antibody.

2.8. Histologic analysis for pancreas inflammatory cell infiltration and vacuolization and measurement of edema.

Quantification of inflammatory cell infiltration and vacuolization was performed on H&E stained pancreatic tissue (7 hourly cerulein injections or L-arginine model) sections from 4–5 mice per group and expressed as the number of inflammatory cells or vacuoles per 100 acinar cells. For cerulein-induced pancreatitis, neutrophil infiltration in pancreas was further quantified in pancreatic tissue sections stained with myeloperoxidase (MPO) antibody (Thermo Fisher Scientific, Rockford, IL) by immunohistochemical staining. Pancreatic edema was evaluated by grading from 0–3 according to Schoenberg grading system [45] and by measuring the wet-to-dry weight ratio as described [46,26].

2.9. Preparation of nuclear extracts and NF-κB DNA binding activity measurement.

Nuclear extracts of the pancreas tissue collected 30min after 1 injection of cerulein were prepared and NF-κB DNA binding activities were measured with ELISA method using Active Motif TRANSAM NFKB P65 kit (Carlsbad, CA) following the manufacturer's instructions.

2.10. Immunofluorescence

Pancreas was dissected and fixed in formalin. The sections were de-paraffinized, rehydrated, and subjected to antigen retrieval by heating. After blocking in 5%Aurion-BSA/0.1%Triton-X100/TBS for 30min at room temperature, the sections were stained with primary antibodies against NF-κB P65 overnight, followed by incubation with secondary antibodies conjugated with Alexa-594 (Life Technologies, Grand Island, NY). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Images were acquired using Leica TCS-SP5 confocal microscope (Leica Microsystems, Buffalo Grove, IL).

2.11. Quantitative Real-time PCR (qPCR) analysis

RNA was isolated from pancreas as described [29]. Reverse transcription was performed with the iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA) using 1μg of total RNA, and the synthesized cDNA samples were used as templates for qPCR analysis following the kit manufacturer's instructions. All reactions were performed using the Bio-Rad CFX ConnectTM qPCR Detection System and the amplifications were done with the iTaq™ Universal SYBR1 Green Supermix (Bio-Rad, Hercules, CA). Gene specific, intron-spanning primers (Table 1) were used. Relative transcript levels were calculated using the comparative 2-ΔΔCt method [47] and normalized to the housekeeping gene 18S rRNA.

2.12. Statistical analysis of data.

Results are means ± SEM and represent data from at least three independent experiments. The experimental data was subjected to two tailed Student t-test for comparison between 2 groups and two-way analysis of variance (ANOVA) followed by post-hoc Tukey tests for comparisons between more than two groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Pancreatic-specific deletion of PKD3

Since the predominant form of PKD in mouse pancreas is PKD3 [41], we developed a mouse model with deletion of this isoform of PKD in the pancreas. As described in Methods, the CreloxP recombination system was used to generate a conditional *Prkd3 (Prkcn*) allele that could be deleted specifically in pancreatic cells. PKD3*Δpanc* mice were developed by crossing *Pkd3flox/flox* mice (referred as PKD3*F/F* mice) with *Pdx1-Cre* transgenic mice which express Cre recombinase under the control of the mouse *Pdx1* promoter.

PKD3*Δpanc* mice were healthy and did not display phenotypic differences (such as size, body weigh) comparing their WT (PKD3*F/F*) littermates. Pancreas-specific truncation of the *Prkd3* gene was confirmed by PCR in homozygotes (PKD3*F/FCre*) and also in heterozygote (PKD3*F/-Cre*) using genomic DNA of pancreas (Fig. 1A: see truncation in 267 bp position) and liver (Fig. 1B: no truncation in 267 bp position). The observed residual un-truncated DNA bands (553 bp) in pancreas from PKD3*F/-Cre* or PKD3*F/FCre* mice (Fig. 1A) were most likely reflecting PKD3*flox* gene in blood cells, blood vascular cells, and immune cells within the pancreas. Deletion of PKD3 protein in pancreas was confirmed by Western blot analyses with PKD3 antibodies from two manufacturers: Cell Signaling (CS, Fig. 1C, **the left panel**) and Bethyl Laboratories (BL, Fig. 1C, **the right panel**). Moreover, we confirmed deletion of PKD3 protein in isolated pancreatic acinar cells (Fig. 1D). Similar to the PCR result in Fig. 1A, we observed residual expression of PKD3 in Western blots of pancreatic tissue lysates from PKD3*Δpanc* mice (either Figure 1C **left or right panel**) but not in the blot of isolated acinar cell lysate from PKD3*Δpanc* mice (Figure 1D), which indicates that residual expression of PKD3 in Western blots of pancreatic tissue lysates from PKD3*Δpanc* mice was most likely reflects PKD3 expression in blood cells, blood vascular cells, and immune cells within the pancreas. The similar results were also observed in mice with cardiac-specific deletion of PKD1 [48].

Quantification of PKD3 mRNA by real-time RT-PCR revealed a 5-fold reduction of PKD3 mRNA in pancreas from PKD3*Δpanc* mice (Fig. 1E). The observed residual expression of PKD3 mRNA also most likely reflects PKD3 expression in blood cells, blood vascular cells, and immune cells within the pancreas.

3.2. Pancreas-specific PKD3 deletion did not affect pancreatic levels of major kinases in the PKD/PKC family

In contrast to PKD3 mRNA, PKD1 and PKD2 mRNA expression in pancreas of PKD3*Δpanc* mice showed similar levels to the control PKD3*F/F* mice (Suppl. Fig. S1A). Western blot analyses showed that pancreatic levels of PKD1 and PKD2, and other major PKCs (PKC epsilon and delta) were similar in wild-type (WT), PKD3*F/F* and PKD3*F/FCre* (PKD3*Δpanc*) mice (Suppl. Fig. S1B), indicating that PKD3 deletion does not affect pancreatic levels of major kinases in PKD/PKC family and no compensatory upregulation of PKD1 and PKD2 genes in PKD3*Δpanc* mice.

3.3. Pancreas-specific PKD3 deletion attenuated necrosis and severity of cerulein pancreatitis

Next, we evaluated the effect of PKD3*Δpan*c on the parameters of cerulein-induced pancreatitis. Cerulein injections caused a marked increase in blood amylase and lipase activities after 7 hourly IP injections of cerulein in WT (PKD3*F/F)* mice. This increase was significantly attenuated in PKD3*Δpan*c mice (Fig. 2). Western blot analyses of pancreatic tissue lysates showed similar amylase or lipase protein expression levels between PKD3*Δpan*c mice and WT mice (Fig. 2A and 2B, **lower panels**), which excluded the possibility that the decreased activities of blood amylase and lipase were caused by the alteration of their expression levels in the pancreas of PKD3*Δpan*c mice.

Importantly, pancreas-specific PKD3 deletion dramatically ameliorated the histological damage in cerulein-induced pancreatitis (Fig. 3A). The first salient feature of the pancreatic histological changes in PKD3*Δpan*c mice was significantly attenuated (by 50%) acinar cell necrosis compared to WT (PKD3*F/F)* mice (Fig. 3B). The other pancreatic injuries in pancreatitis including accumulation of cytoplasmic vacuoles and edema were also significantly reduced in PKD3*Δpan*c mice (Fig. 3C–E). We measured edema with edema score and the wetto-dry weight ratio of pancreatic tissue (Fig. 3D, E). Both measurements indicated that the edema was significantly reduced in pancreatitis in PKD3*Δpan*c mice. These results demonstrate that PKD3 deletion ameliorates necrosis and the severity of acute pancreatitis.

3.4. Pancreas-specific PKD3 deletion attenuated inflammatory cell infiltration in cerulein pancreatitis

To evaluate the effect of pancreas specific PKD3 deletion on inflammatory cell infiltration in cerulein pancreatitis, immunohistochemical staining of myeloperoxidase (MPO) were performed on pancreatic tissue sections from PKD3*F/F* and PKD3Δ*panc* mice using specific MPO antibody (Fig. 4A, B). Cerulein treatment markedly increased numbers of MPO staining positive cells (infiltrated immune cells) in the pancreas from WT (PKD3*F/F)* mice, whereas MPO staining positive cells (dark brown cells) were significantly less in PKD3*Δpan*c mice. These results were consistent with inflammatory cell infiltration counted from H&E stained sections (Fig. 4C).

3.5. Pancreas-specific PKD3 deletion inhibited NF-κB activation and nuclear translocation and IκB-α degradation in cerulein-induced pancreatitis.

The nuclear transcription factor NF-κB has been known as a key signal triggering inflammation during pancreatitis [9–17]. Our previous studies using molecular approach and specific PKD pharmacologic inhibitors in *in vitro* and *in vivo* rat pancreatitis models demonstrated that PKDs were required for NF-κB activation in pancreatitis [40,42]. Here, we showed that genetic deletion of the PKD isoform, significantly decreased NF-κB activation in cerulein-induced pancreatitis (Fig. 5A), supporting the critical role of PKD members in NF-κB activation in acute pancreatitis.

Translocation from cytosol to nucleus is a characteristic of activated NF-κB. Nucleus translocation of NF-κB subunits results in transcriptional activation of a number of pro-

inflammatory genes in pancreatic acini [11]. We used two approaches to determine nucleus translocation of NF-κB: subcellular fractionation and immunostaining of the pancreatic tissue collected in mouse cerulein pancreatitis. Comparing NF-κB P65 level in cytosolic fractions from PKD3*F/F* mice and PKD3Δ*panc* mice (Fig. 5B), we found that NF-κB was present predominantly in the cytosolic fraction of pancreas from either strain of mice injected with saline. Cerulein treatment almost abolished the presence of NF-κB in the cytosolic fraction in PKD3*F/F* mice. But there is still the presence of some NF-κB in the cytosolic fraction in PKD3Δ*panc* mice. Moreover, our Western blot analysis of pancreatic cytosol also showed that deletion of PKD3 prevented degradation of IκB-α, the inhibitory protein of NF-κB, in cerulein-induced pancreatitis (Fig. 5B). The inhibitory effect of PKD3 deletion on either nuclear translocation of NF-κB or IκB-α degradation was in accordance with its inhibitory effects on NF-κB-DNA binding activity (Fig. 5A, B).

The nuclear translocation of NF-κB P65 was further confirmed by immunofluorescence staining of the pancreatic tissue sections. Confocal immunofluorescence (Fig. 6) showed that in saline-treated mice, the staining of NF-κB (shown in red) distributed throughout the cytosol in both PKD3*F/F* mice and PKD3Δ*panc* mice. Upon cerulein administration, we observed dramatically increased colocalization of NF-κB P65 (red) and nucleus (blue) in PKD3*F/F* mice, but the colocalization of NF-κB P65 and nucleus was much less in PKD3Δ*panc* mice (Fig. 6).

3.6. NF-κB Inhibition in mice with pancreas-specific PKD3 deletion was associated with decreased pancreatic mRNA expression of inflammatory cytokines and chemokine.

To test whether the inhibition of NF-κB by genetic inhibition of PKD3 altered mRNA expression of inflammatory molecules in pancreatitis, we examined pancreatic level of cytokine IL-6, IL-1β, TNF-α and chemokine MCP-1 (Fig. 7A–D) since promoters of these genes have binding sites of NF-κB [10–12,17]. All these four inflammatory molecule mRNA were very low within the pancreas from saline-treated mice and significantly increased in PKD3*F/F* mice administrated with cerulein. However, the cerulein-caused high mRNA expression of these inflammatory molecules were dramatically reduced in PKD3Δ*panc* mice (Fig. 7A–D).

3.7. Pancreas-specific PKD3 deletion did not affect CCK-induced amylase secretion but attenuated CCK-induced LDH and HMGB1 release in pancreatic acinar cells.

Using isolated acini from PKD3Δ*panc* mice and PKD3*F/F* control mice, we measured CCK-stimulated amylase release over a concentration range of CCK (0.001–100 nmol/L; Fig. 8A). In both PKD3*F/F* control mice and PKD3Δ*panc* mice, amylase release peaked at 0.01nM CCK and subsequently decreased. No significant difference was seen in amylase release between the two sets of mice.

To corroborate the findings that PKD3 promoted necrosis in the *in vivo* model of pancreatitis, we performed experiments on isolated pancreatic acinar cells. The acinar cells were stimulated with supramaximal (100nM) CCK which was known to induce pancreatitis responses in acinar cells [31,29,40,25]. Therefore, this system was considered as *in vitro* model of acute pancreatitis.

Pancreatic acini isolated from PKD3*F/F* mice and PKD3Δ*panc* mice were hyperstimulated with CCK (100nM). Changes in LDH or HMGB1 release into the extracellular medium were measured after 3 hr incubation with CCK. We observed that CCK hyperstimulation-caused LDH release, an indicator of cell necrosis, was significantly less in PKD3Δ*panc* acini than in PKD3*F/F* acini (Fig. 8B). Consistent with this result, CCK also induced much high release of HMGB1, an inflammatory mediator released by necrotic cells, in PKD3*F/F* acini than in PKD3Δ*panc* acini (Fig. 8C). All these findings suggest that genetic inhibition of PKD3 protects the acinar cells from CCK-induced necrosis.

3.8. Pancreas-specific PKD3 deletion promoted degradation of RIP1 in cerulein-induced pancreatitis.

Our previous studies showed that PKD inhibition by chemical inhibitor promoted RIP1 cleavage/inactivation, accounting for the ameliorated effect of PKD inhibition on necrosis [25]. To explore the pathways mediating the role of PKD3 in pancreatic necrosis in pancreatitis, here we determined the effect of the genetic deletion of PKD3 on RIP1 degradation (Fig. 9A). In agreement with previous findings [23,26], RIP1 remained at a relatively stable level during cerulein-induced pancreatitis in WT (PKD3*F/F*) mice. Of note, the expression level of RIP1 in control PKD3Δ*panc* mice injected with saline was similar to that in PKD3*F/F* mice. However, RIP1 underwent a dramatic reduction (~50–60%) in PKD3Δ*panc* mice after seven hourly cerulein injections (Fig. 9A). The results suggest that PKD3 deletion promotes RIP1 degradation/cleavage, and this may be one of mechanisms accounting attenuated necrosis in pancreatitis in PKD3Δ*panc* mice.

3.9. Pancreas-specific PKD3 deletion prevented pancreatic ATP depletion in pancreatitis and caused a more prominent upregulation of pancreatic mitochondrial prosurvival Bcl-2 proteins and mRNA expression.

We compared changes in pancreatic ATP levels in pancreatitis between the two strain mice. ATP was dramatically depleted (by ~60%) in cerulein-induced pancreatitis in WT (PKD3*F/F*) mice, whereas pancreatic ATP production was only little reduced (~10%) in PKD3Δ*panc* mice (Fig. 9B), indicating that genetic inhibition of PKD3 protected pancreas from ATP depletion in pancreatitis.

To further provide insights into the mechanism of protection against cell necrosis in PKD3Δ*panc* mice, we next analyzed pancreatic Bcl-2 and Bcl-xL, two prosurvival Bcl-2 proteins majorly localized to the outer membrane of mitochondria, where they play an important role in stabilizing pancreatic mitochondria and protecting against necrosis in pancreatitis. Up-regulation of Bcl-2 and Bcl-xL has been demonstrated as a key protective mechanism against necrosis in pancreatitis [26,29,30].

We found that upregulation of Bcl-2 and Bcl-xL proteins in pancreatitis was more pronounced in PKD3Δ*panc* mice than in PKD3*F/F* mice (Fig. 9C, D). Consistent with their protein levels, the mRNA expression of Bcl-2 and Bcl-xL was also more increased in PKD3Δ*panc* mice compared to PKD3*F/F* mice (Fig. 9E, F). The greater extent of upregulation of the prosurvival Bcl-2 and Bcl-xL proteins in PKD3Δ*panc* may account, in part, for necrosis attenuation in pancreatitis in PKD3Δ*panc* mice and suggests that the

negative regulation of Bcl-2 and Bcl-xL proteins by this PKD isoform might be a mechanism whereby PKD3 promotes necrosis in pancreatitis.

3.10. Pancreas-specific PKD3 deletion promoted caspase activation in cerulein-induced pancreatitis

In previous studies, we investigated the role and mechanisms of PKD/PKD1 in the regulation of cell death in experimental models of acute pancreatitis by using PKD inhibitors CID755673. Our results demonstrated that PKD inhibition by the chemical inhibitor attenuated necrosis while promoting apoptosis/caspase activation in pancreatitis [25]. Here, to determine the role of PKD3 in apoptosis, we measured activation of caspase 3, the major effector caspase mediating cell apoptosis, in cerulein-induced pancreatitis. Interestingly, the pancreatic caspase 3 activity was significantly enhanced (by 4-folds over the saline control) in pancreatitis in PKD3*Δpanc* mice compared in the control PKD3*F/F* mice (Suppl. Fig. S2), suggesting that genetic inhibition of PKD3 stimulated caspase activation / apoptosis in pancreatitis. This result supports our previous studies with pharmacological PKD inhibitors [25].

3.11. Pancreas-specific PKD3 deletion attenuated pancreatic trypsinogen activation and cathepsin B activity in cerulein pancreatitis

Using pharmacologic PKD inhibitors, we previously demonstrated that PKD/PKD1 mediates trypsinogen activation in acute pancreatitis [31,42]. Here, we found that cerulein-induced intrapancreatic trypsinogen conversion to trypsin (as measured by trypsin activity) was markedly reduced in PKD3Δ*panc* mice (Fig. 10A), Western blot analyses of pancreatic tissue lysates showed basal trypsinogen protein expression levels before cerulein treatment were similar between PKD3Δ*panc* mice and PKD3*F/F* mice (Fig. 10A, **lower panel**), which excluded the possibility that the decreased activities of pancreatic trypsin were caused by the alteration of trypsinogen basal expression levels in the pancreas of PKD3Δ*panc* mice.

We further explored the potential mechanism by which PKD3 influences zymogen activation. The lysosomal hydrolase cathepsin B is largely responsible for activation of trypsinogen. We found that cerulein-induced cathepsin-B activity was also inhibited correspondingly by ~50% in PKD3Δ*panc* mice, indicating that PKD3 regulates cathepsin B-dependent trypsinogen activation (Fig. 10B).

3.12. Pancreas-specific PKD3 deletion enhanced autophagy flux in cerulein pancreatitis

Disorder in autophagy participates in the intracellular trypsin activation and the other pathologic responses in pancreatitis [32,34,35]. To assecess whether PKD isoform mediate autophagy flux in pancreatitis, we examined the expression levels of proteins involved in autophagy (Fig. 11). Cerulein treatment increased the autophagy-related proteins LC3-II and p62 levels in PKD*F/F* mice. However, in PKD3*Δpanc* mice, cerulein-increased LC3-II levels were significantly higher compared to PKD3*F/F* mice, without any significant difference in p62 levels (Fig. 11A–C). Furthermore, cerulein treatment significantly reduced the phosphorylation level of ULK1 (S555) and AMPK (T172), the proteins involved in autophagosome-lysosome fusion process, in control PKD3*F/F* mice but not in PKD3*Δpanc* mice (Fig. 11A, D, E). These results suggest that the fusion process was impaired in

pancreatitis in PKD3*F/F* mice but restored in PKD3*Δpanc* mice through activation of AMPK-ULK1 complex.

3.13. Pancreas-specific PKD3 deletion attenuated necrosis, inflammation, and severity of L-arginine-induced pancreatitis

To further determine that PKD plays an important role in pancreatitis, we performed another experimental model, L-arginine-induced pancreatitis in PKD3*Δpan*c mice and the control PKD*F/F* mice. Consistent with cerulein pancreatitis model, pancreas-specific PKD3 deletion also dramatically ameliorated pancreatitis parameters including serum amylase activation, pancreatic trypsinogen activation, and histological damage in arginine-induced pancreatitis (Fig. 12A–C). We observed significant attenuation in pancreatic necrosis (by ~70%, Fig. 12D), inflammatory cell infiltration (by ~ 40%, Fig. 12E), and edema (by ~ 60%, Fig. 12F) in PKD3*Δpan*c mice. Moreover, we examined pancreatic mRNA expression of cytokine IL-6, IL-1β, TNF-α and chemokine MCP-1 (Fig. 13A–D). All these four inflammatory molecule mRNAs were dramatically reduced in PKD3Δ*panc* mice in arginine pancreatitis (Fig. 13A–D). Thus, with the second experimental pancreatitis model, we demonstrated again that PKD3 deletion ameliorated inflammation, necrosis, and the severity of acute pancreatitis.

4. Discussion

We have generated a mouse model with a pancreas specific deletion of PKD3, the predominant PKD isoform in mouse pancreatic acinar cells. Pancreas-specific truncation of the PKD3 gene and resulted inhibition in expression of PKD3 mRNA and protein were confirmed by PCR, qPCR and Western blot analysis. PKD3 deletion does not affect pancreatic levels of major kinases in the PKD/PKC family. Similar to the control mice PKD3*F/F*, PKD3*Δpan*c mice are healthy and do not display phenotypic (such as size, body weight) or histological abnormalities in pancreas or other organs. They have normal acinar cell amylase secretion, content of pancreatic digestive enzymes, fecal output, behavior, and reproductive capability.

PKD3*Δpan*c mice displayed significant attenuation in inflammation, acinar cell necrosis, trypsinogen activation, and severity of pancreatitis in two experimental models of acute pancreatitis. These novel findings substantiate our previous results with pharmacologic inhibitors and confirm that PKDs play a necessary role in key pathobiological processes of pancreatitis.

With cerulein pancreatitis model, we explored the mechanism underlying the effect of pancreas-specific PKD3 deletion on pancreatitis, we first investigated the nuclear transcription factor NF-κB, a key signal triggering inflammation during pancreatitis [9–17]. NF-κB P65 activation and nucleus translocation in pancreatitis were both significantly decreased in PKD3*Δpan*c mice. Degradation of IκB-α, the inhibitory protein of NF-κB, also correspondingly decreased in PKD3*Δpan*c mice. Furthermore, we found that NF-κB Inhibition in PKD3*Δpan*c mice resulted in, sequentially, decreased pancreatic mRNA expression of several important inflammatory mediators including cytokines IL-6, IL-1β, TNF-α and chemokine MCP-1, less infiltration of inflammatory cells, and ameliorated

edema in pancreatitis. These findings support our previous results achieved with either molecular approach or pharmacological PKD inhibitors [40,31,42] indicating the role of PKD in NF-κB activation and inflammatory response in pancreatitis.

In addition to the attenuation effect of PKD3 deletion on inflammatory response of pancreatitis, we demonstrated that pancreas specific PKD3 deletion significantly ameliorated necrosis in pancreatitis in both *in vivo* and *in vitro* experimental models of pancreatitis. Pancreas-specific PKD3 deletion dramatically ameliorated pancreatic necrosis (by 50%) in cerulein-induced *in vivo* mouse pancreatitis. Consistent with this result, we showed that CCK hyperstimulation-caused LDH or HMGB1 release to the extracellular medium in isolated PKD3Δ*panc* acini was also markedly decreased. All these findings suggest that genetic inhibition of PKD3 protects the acinar cells from necrosis in pancreatitis.

Furthermore, to determine the role of PKD3 deletion in apoptosis, we examined pancreatic caspase 3 activity and found that caspase 3 activation was significantly enhanced in PKD3Δ*panc* mice in pancreatitis, suggesting that pancreas-specific deletion of PKD3 stimulated caspase activation/apoptosis in pancreatitis. The role of PKD3 deletion promoting apoptosis is one of important contributors to attenuate pancreatitis in PKD3Δ*panc* mice because the severity of acute pancreatitis correlates with the extent of necrosis, and inversely correlates with apoptosis as shown in different studies in experimental pancreatitis [23,25,29,49].

Our earlier studies found that PKD1 played a role in pathological dose secretagogue-induced amylase secretion in pancreatitis [31]. Pretreatment of isolated rat pancreatic acinar cells (which predominantly express PKD1 isoform) with PKD inhibitor CRT0066101 did not affect amylase secretion induced by physiological concentrations of CCK but significantly inhibited high dose CCK (100nM)-induced amylase secretion in pancreatitis [31]. The current study with PKD3Δ*panc* mice showed that compared with the control WT mice (PKD3*F/F*), genetic inhibition of PKD3, the predominant PKD isoform in mouse pancreatic acini, did not affect amylase secretion stimulated with a concentration range of CCK from physiological dose to hyperstimulation dose (0.001–100 nM), indicating that the deletion of the PKD3 isoform did not affect amylase release. But, different from PKD1 isoform, PKD3 did not play a role in high dose-stimulated amylase secretion, suggesting an isoform-specific function in PKD family. Our findings also confirmed the result from another group [41] that PKD3 knockdown in acinar cells by lentiviral-mediated RNA interference failed to inhibit CCK stimulated amylase release, indicating that PKD3 alone was not required for amylase secretion. Our results demonstrated that the deletion of the PKD3 isoform did not affect pancreatic exocrine functionality.

RIP1 has been known as a key mediator of programmed necrosis in many diseases including acute pancreatitis [19–26]. RIP1 protein level decreased through degradation/cleavage during pancreatitis. Our earlier studies using PKD inhibitors demonstrated PKD promoted necrosis in cerulein-induced pancreatitis through mechanisms involving RIP1 [25]. Furthermore, we found that genetic deletion of PKCε, a major upstream kinase regulator of PKD, promoted degradation/inactivation of RIP1 in pancreatitis in the mouse, resulting in attenuation of necrosis and severity of pancreatitis [26]. Here, we showed that genetic

deletion of PKD3 resulted in significantly decreased RIP1 protein level in cerulein-induced pancreatitis. This finding supports our previous results obtained with pharmacologic PKD inhibitors and genetic inhibition of PKCε mouse model [25,26] and confirms that PKD-mediated prevention of RIP1 cleavage may be one of mechanisms underlying the exacerbating effect of PKD on necrosis in acute pancreatitis.

Evidence [26–29] from our studies and other groups indicated that necrosis in pancreatitis was associated with ATP depletion. Thus, here we assessed the effect of genetic inhibition of PKD3 on mitochondrial ATP depletion in pancreatitis. The results showed that ATP depletion in pancreatitis was dramatically prevented in PKD3Δ*panc* mice, suggesting an important role for PKD3 in regulating mitochondrial ATP production in pancreatitis. It has been increasingly recognized that the production of ATP is one of important factors regulating pancreatic cell death [49–53]. The ability of autophagy to defend cells against various stressors is obtained partly through the generation of substrates to maintain ATP production [50]. Intracellular ATP levels determine cell death fate [49,51,52]. ATP depletion leads to necrosis, whereas ATP production is required for the induction of apoptosis [49]. Furthermore, some reports observed that intracellular administration of ATP could abolished acinar injury in response to ethanol metabolites [51,52]. Thus, an effective ATP delivery system combined with the prevention of further mitochondrial damage may open up the possibility of pharmacological therapy for acute pancreatitis, leading to reduced disease severity and mortality [53]. Our results showing the effect of PKD3 on bioenergetics suggest that preventing ATP depletion through inhibiting PKD might be a strategy for treatment of acute pancreatitis.

We further analyzed pancreatic expression of Bcl-2 and Bcl-xL, two members of mitochondrial prosurvival Bcl-2 proteins. Previous studies in our group [29,30] indicated that these two Bcl-2 proteins could stabilize pancreatic mitochondria membrane permeability and maintain mitochondrial functions including energy metabolism and ATP production, but inhibition of Bcl-2 and Bcl-xL exacerbated mitochondrial ATP depletion and cell necrotic death in pancreatitis. Thus, up-regulation of Bcl-2 and Bcl-xL has been considered as an important protective mechanism against necrosis in pancreatitis. In current study, we found more prominent upregulation of the pancreatic prosurvival Bcl-2 proteins in cerulein-induced pancreatitis in PKD3Δ*panc* mice, which may have provided the protection against cell necrosis.

Intra-pancreatic activation of trypsinogen is involved in pancreas injury and pancreatitis. Using small molecule PKD inhibitors, we previously found that PKD regulated lysosomal hydrolase cathepsin B-induced trypsinogen activation and PKD inhibition decreased pancreatic trypsinogen activation through cathepsin B in pancreatitis. Our present studies demonstrated that both pancreatic trypsinogen activation and cathepsin B activation in pancreatitis were inhibited in PKD3Δ*panc* mice, confirming that PKD regulates cathepsin B-induced trypsinogen activation. Trypsinogen activation and NF-κB activation are all early events that contribute to the pathologic process of acute pancreatitis. Our results suggest that PKD family is a potential target for therapy in the early stage of the disease.

Impaired autophagosome-lysosome fusion in process of autophagy has been recognized as a key pathologic response occurring in pancreatitis. The disorder of autophagy flux can contribute to pathologic process of pancreatitis including pancreatic vacuolization, trypsinogen activation, cell death and inflammation [34,35]. The functions of PKD family members in autophagy flux in pancreatitis remain to be studied. We here preliminarily investigated whether pancreatic specific deletion of PKD3 effects on proteins involved in autophagy flux in pancreatitis. The results showed that the autophagy flux impaired in cerulein-induced pancreatitis was improved/restored in PKD3Δ*panc* mice, namely, the inhibition of PKD3 was associated with reversing the inhibition of AMPK and ULK-1 that occurs during pancreatitis, implying that PKD3 may regulate autophagy in pancreatitis through negatively modulating the activation of AMPK and ULK-1. However, current knowledge of the negative regulation of AMPK and ULK-1 by PKD3 is limited, further studies are needed in future. Neverthless, our findings may reveal the novel role of PKD inhibition in enhancing autophagic flux to prevent pancreatic cell injury and pancreatitis. It is necessary in future to elucidate the molecular mechanism involved in regulation of autophagy by PKD3.

Finally, to substantiate our results achieved in the cerulein model for role of PKD in pancreatitis, we evaluated the effect of PKD3*Δpan*c on L-arginine-induced pancreatitis. As we expected, pancreas-specific deletion of PKD3 also dramatically ameliorated inflammation, necrosis, cell injury, and the severity of pancreatitis. Arginine pancreatitis associated high mRNA expression of inflammatory cytokine and chemokine were significantly suppressed in PKD3*Δpan*c mice. Thus, with the second experimental pancreatitis model, we further demonstrated the important role of PKD in pancreatitis.

5. Conclusions

We have generated a mouse model with a pancreas specific deletion of PKD3 (PKD3*Δpan*c) The PKD3*Δpan*c mice displayed significant attenuation in inflammation, necrosis, and severity of pancreatitis in two experimental pancreatitis models. As we summarized in Fig. 14, activation of PKD3 in pancreatitis promoted inflammation and necrosis, the two pathological features of this disorder, through enhancing NF-κB mediated inflammatory response and regulating the cell death factors including mitochondrial ATP production, Bcl-2 family proteins, and RIP1 kinase, as well as trypsinogen activation and autophagy related proteins. With this genetic mouse model of pancreas-specific deletion of PKD isoform, we further demonstrated that PKDs play a critical role in pathobiological process of pancreatitis and PKDs constitute a novel therapeutic target to treat this disorder.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Generation of a novel mouse model with pancreas-specific deletion of PKD3 (PKD3*Δpanc* mice)
- PKD3*Δpanc* mice displays attenuation in inflammation, necrosis, and severity of pancreatitis in two experimental pancreatitis models.
- PKD3*Δpanc* mice has decreased NF-κB and trypsinogen activation and pancreatic mRNA expression of inflammatory molecules in pancreatitis.
- PKD3*Δpanc* mice show less pancreatic ATP depletion and RIP1 level but increased pro-survival Bcl-2 proteins, and autophagy promotion in pancreatitis.
- PKD may constitute a novel therapeutic target to treat this disorder.

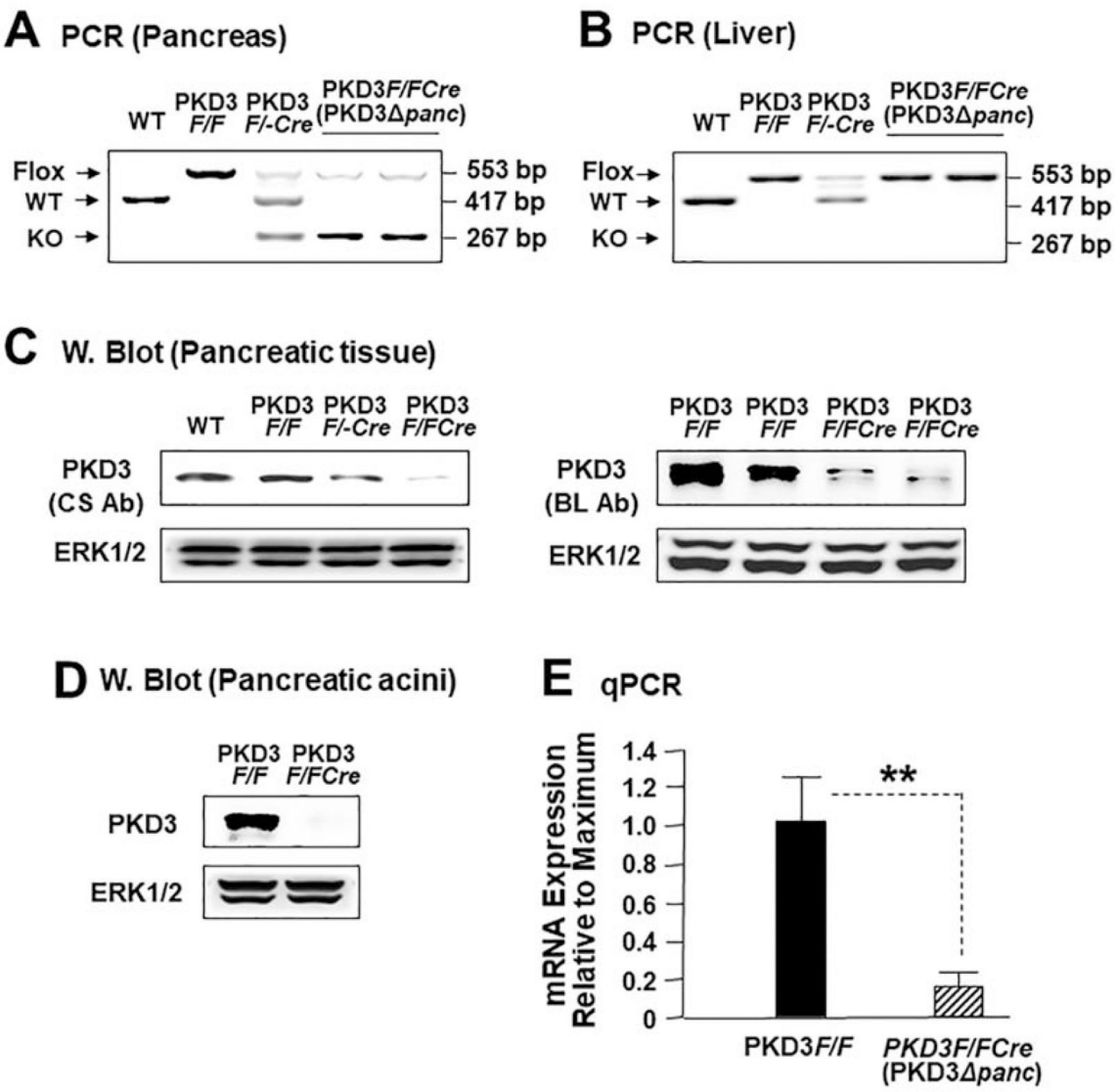

Fig. 1.

Generation of mice with pancreas-specific deletion of PKD3. Pancreas-specific truncation of the *Pkd3* gene was confirmed by PCR in homozygotes (PKD3*F/FCre*) and also in heterozygote (PKD3*F/-Cre*) using genomic DNA of pancreas (A: see truncation in 267 bp position) and liver (B: no truncation in 267 bp position). (C) Deletion of PKD3 protein in pancreatic tissue was confirmed by Western blot analysis with PKD3 antibody purchased from Cell Signaling (C, left panel) and from Bethel Laboratories (C, right panel). (D) Deletion of PKD3 protein in isolated pancreatic acinar cells was confirmed by Western blot analysis with PKD3 antibody. (E) qPCR of pancreatic PKD3 mRNA expression in PKD3*F/FCre* (PKD3Δ*panc*) mice and PKD3*F/F* control mice. Values are means ± SEM (n>6), $^{**}P < 0.01$ versus PKD3*F/F* control mice.

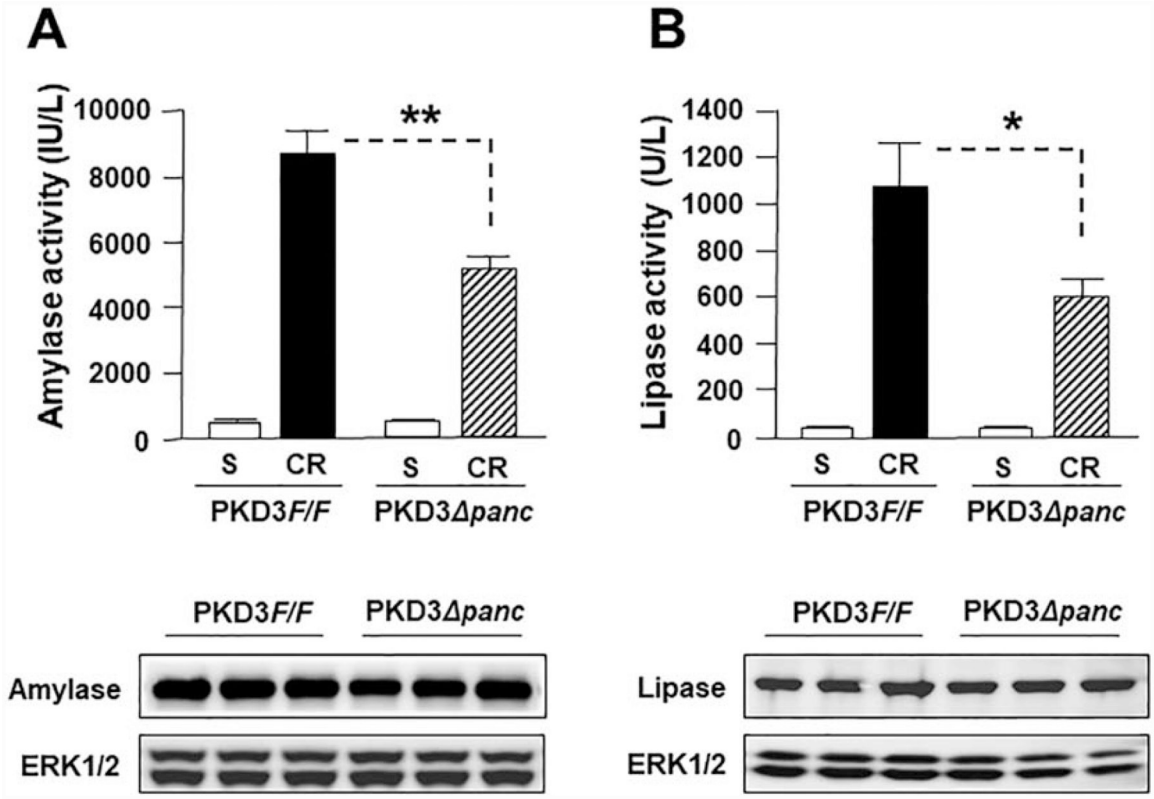

Fig. 2.

Pancreas-specific PKD3 deletion decreased blood amylase and lipase activities in pancreatitis. Serum amylase activity (A) and lipase activity (B) at 7 hourly cerulein injections. Values are means ± SEM (n>8), $^{**}P < 0.01$ or $^{*}P < 0.05$ versus PKD3*F/F* control mice with cerulein; Western blot analysis (*Lower panels*) shows similar pancreatic expression levels of amylase or lipase in PKD3*F/F* and PKD3Δ*panc* mice.

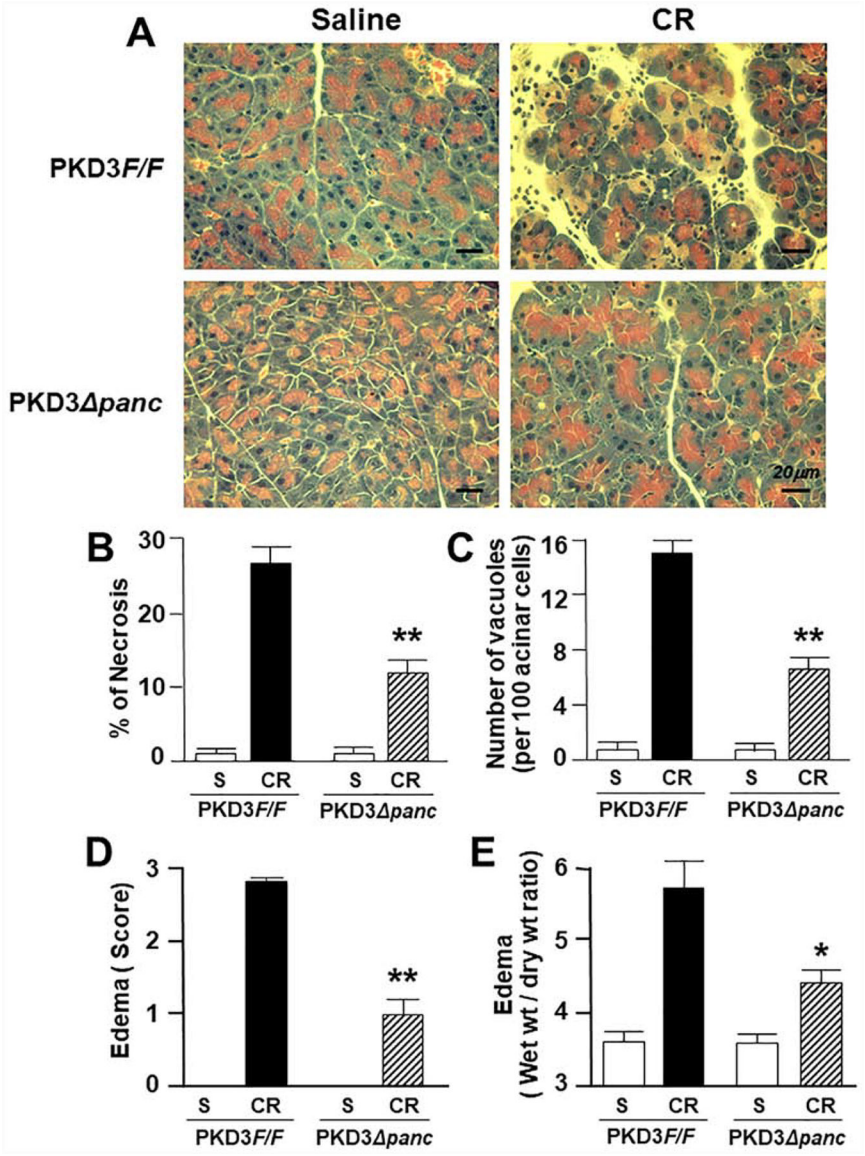

Fig. 3.

Pancreas-specific PKD3 deletion attenuated necrosis and severity in cerulein pancreatitis. (A) H&E staining of pancreatic tissue sections from mice with 7 hourly cerulein (or saline) injections. Each image was representative of at least 4 mice with similar results at each condition. Bars represent 20μm. (B) Necrosis was measured on H&E stained pancreatic tissue sections, as described in Methods. (C) Number of vacuoles was counted on H&E stained pancreatic tissue sections and expressed as percentage of total acinar cells. (D) Pancreatic edema grading was made from 1 to 3 on the H&E stained tissue sections with Schoenberg grading system. 0: no edema; 1: interlobular edema; 2: moderate interlobular and intra-acinar edema; and 3: severe interlobular and intra-acinar edema. (E) Pancreatic edema (water content) was further evaluated by measuring the wet-to-dry weight ratio of the pancreas tissue samples. Results in graphs B-E represent means ± SEM (n=4–6 mice) for

each condition. $^{**}P < 0.01$ or $^{*}P < 0.05$ versus PKD3*F/F* with cerulein injections as indicated.

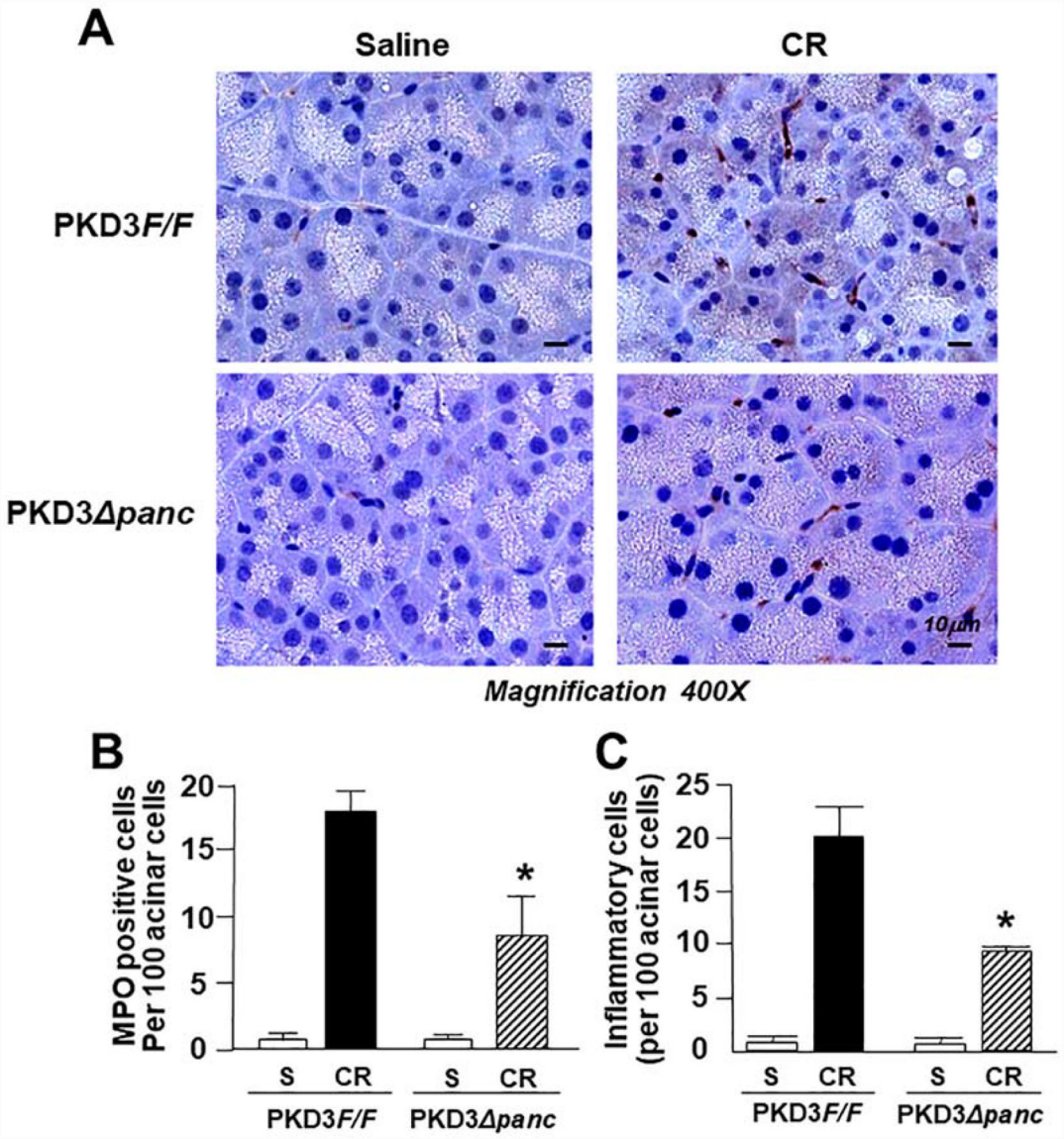

Fig. 4.

Pancreas-specific PKD3 deletion attenuated inflammatory cells infiltration in cerulean pancreatitis. (A) Neutrophil infiltration within pancreas was assessed by immunohistochemical staining of myeloperoxidase (MPO) on pancreatic tissue sections from PKD3*F/F* and PKD3Δ*panc* mice using specific MPO antibody. The dark brown stained cells in exocrine pancreas were determined as MPO positive cells. Bars represent 10 μm. (B) MPO positive cells were counted and expressed as percentage of total acinar cells. (C) Number of inflammatory cells were also counted on H&E stained pancreatic tissue sections and expressed as percentage of total acinar cells. Results in graphs (B-C) represent means ± SEM (n=3–5 mice) for each condition. **P* < 0.05 versus PKD3*F/F* control mice with cerulein injections as indicated.

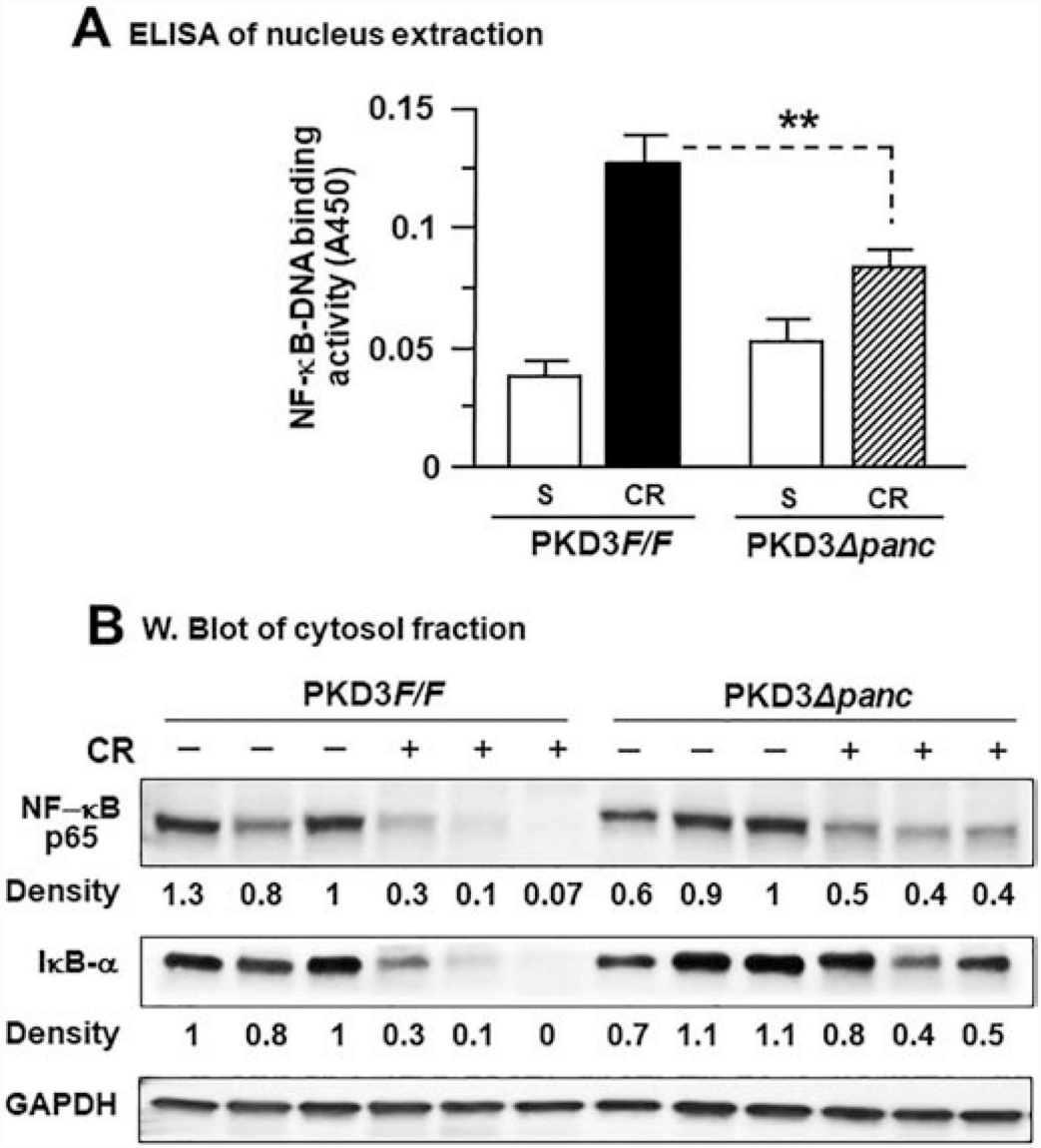

Fig. 5.

Pancreas-specific PKD3 deletion inhibited NF-κB activation and nuclear translocation and IκB-α degradation in cerulein pancreatitis. (A) NF-κB activation was significantly attenuated in PKD3*Δpanc* mice in pancreatitis. Values are means ± SEM (n>6), ** $P < 0.01$ versus cerulein (CR) in PKD*F/F* control mice. (B) Western blot analysis of pancreatic tissue cytosol fraction indicated that NF-κB nuclear translocation and IκB-α degradation were reduced in PKD3*Δpanc* mice in pancreatitis. Each lane represents an individual mouse, n=3. The protein band densities of NF-κB and IκB-α were quantified and normalized to the loading control GAPDH.

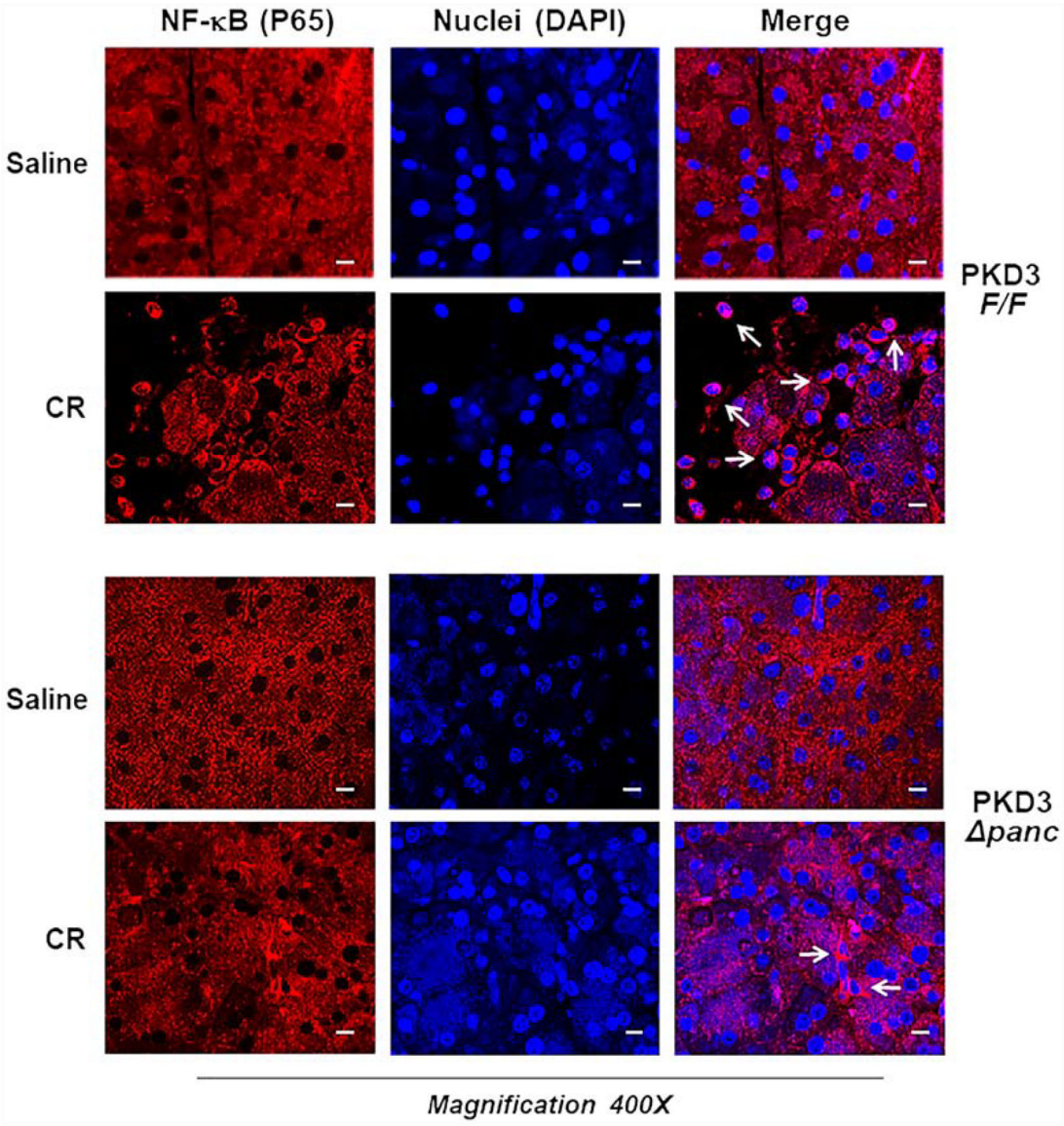

Fig. 6.

Immunofluorescence staining of pancreatic tissue sections with NF-κB P65 antibody. Pancreatic tissue sections were immunostained for NF-κB P65 and secondary antibodies conjugated with Alexa-594 (red). Nuclei were counterstained with DAPI (blue). Images were visualized under confocal microscope. The original magnification is x40. Bar represents 20μm. Images shown were representative of >2 mouse pancreatic tissue sections and indicated that pancreas-specific PKD3 deletion inhibited NF-κB nuclear translocation in cerulein pancreatitis. Arrows: indicate the nucleus (blue)-translocated NF-κB (red).

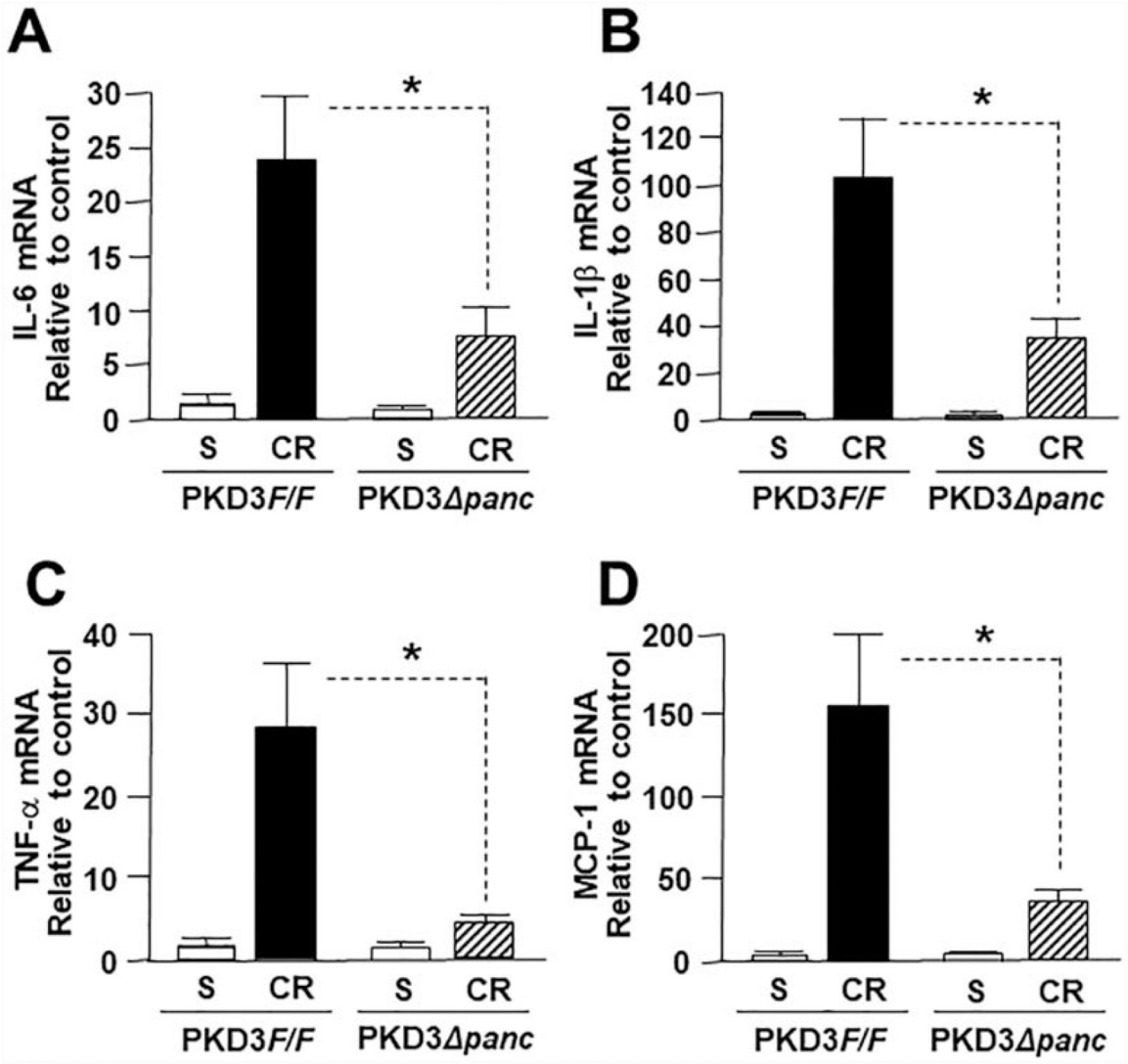

Fig. 7.

Pancreas-specific deletion of PKD3 decreased mRNA expression of Inflammatory molecules. mRNA extracts from pancreas of PKD3*F/F* and PKD3*Δpanc* mice were analyzed by real-time quantitative PCR to determine the expression of IL-6 (A), IL-1β (B), TNF-α (C), and MCP-1 (D) in cerulein (CR)-induced pancreatitis. Bar values are means ± SEM (n>5), **P* < 0.05 versus PKD3*F/F* control mice with CR.

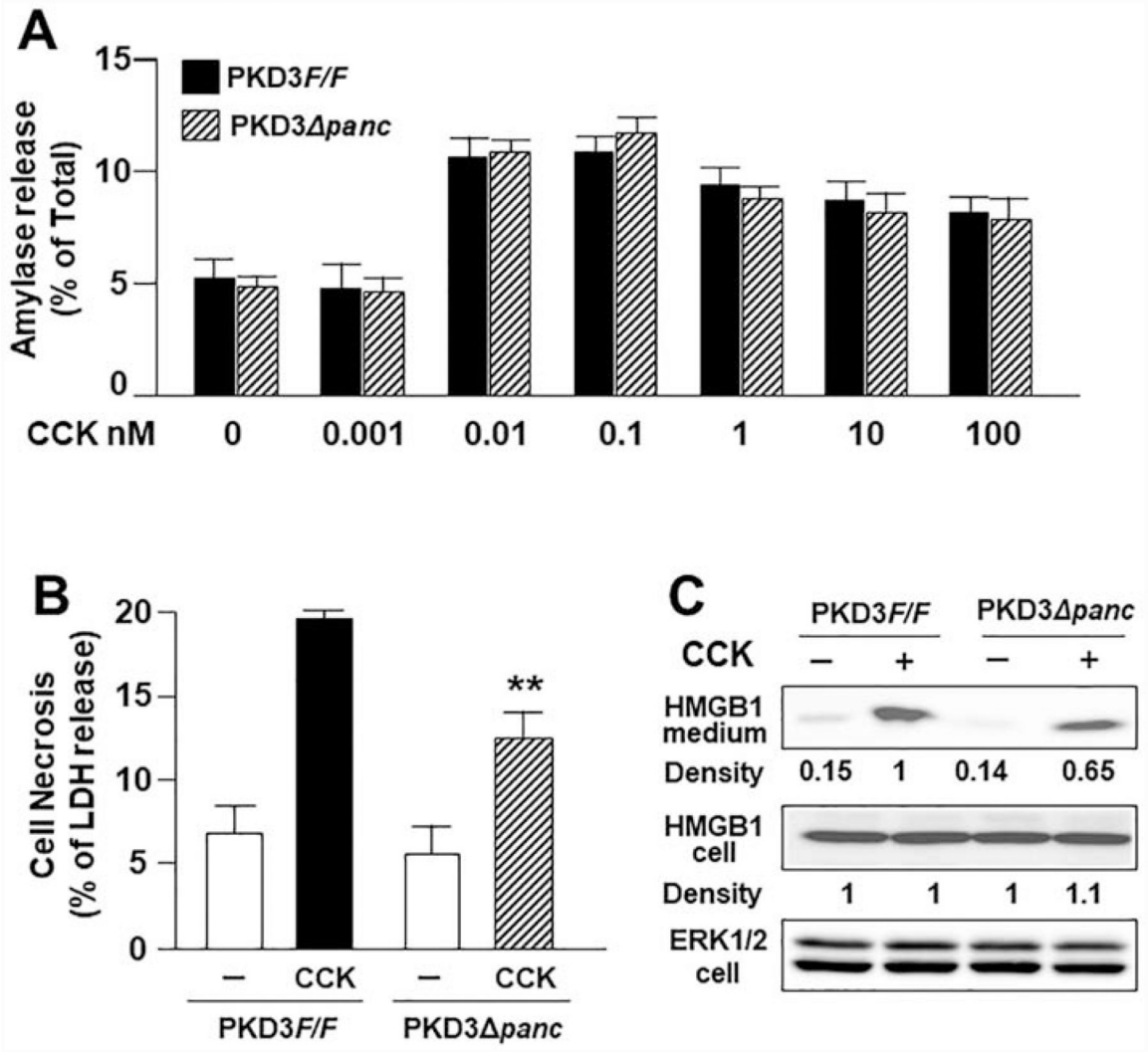

Fig. 8.

Pancreas-specific deletion of PKD3 did not affect CCK-induced amylase secretion but reduced pathologic dose CCK-induced release of LDH and HMGB1 in mouse pancreatic acinar cells. Pancreatic acinar cells were isolated from PKD3*F/F* and PKD3Δ*panc* mice and then stimulated for 30 minutes with CCK (0.001–100 nM) as indicated (A). Amylase release is expressed as percent total release (medium / [medium + cells]). n = 5. For measurement of CCK-induced LDH or HMGB1 release, pancreatic acini isolated from PKD3*F/F* and PKD3Δ*panc* mice were incubated for 3hr without or with 100 nM CCK-8, and then the media and cells were collected separately. (B) percentage of LDH released into the extracellular medium. Values are means ± SEM (n=4). * *P* < 0.01 versus PKD3*F/F* acinar cells incubated with CCK (black bar) as indicated. (C) representative immunoblot images of protein levels of the high-mobility group protein 1 (HMGB1) in media and cell homogenates. Extracellular signal regulated kinase (ERK) 1/2 was used as loading control.

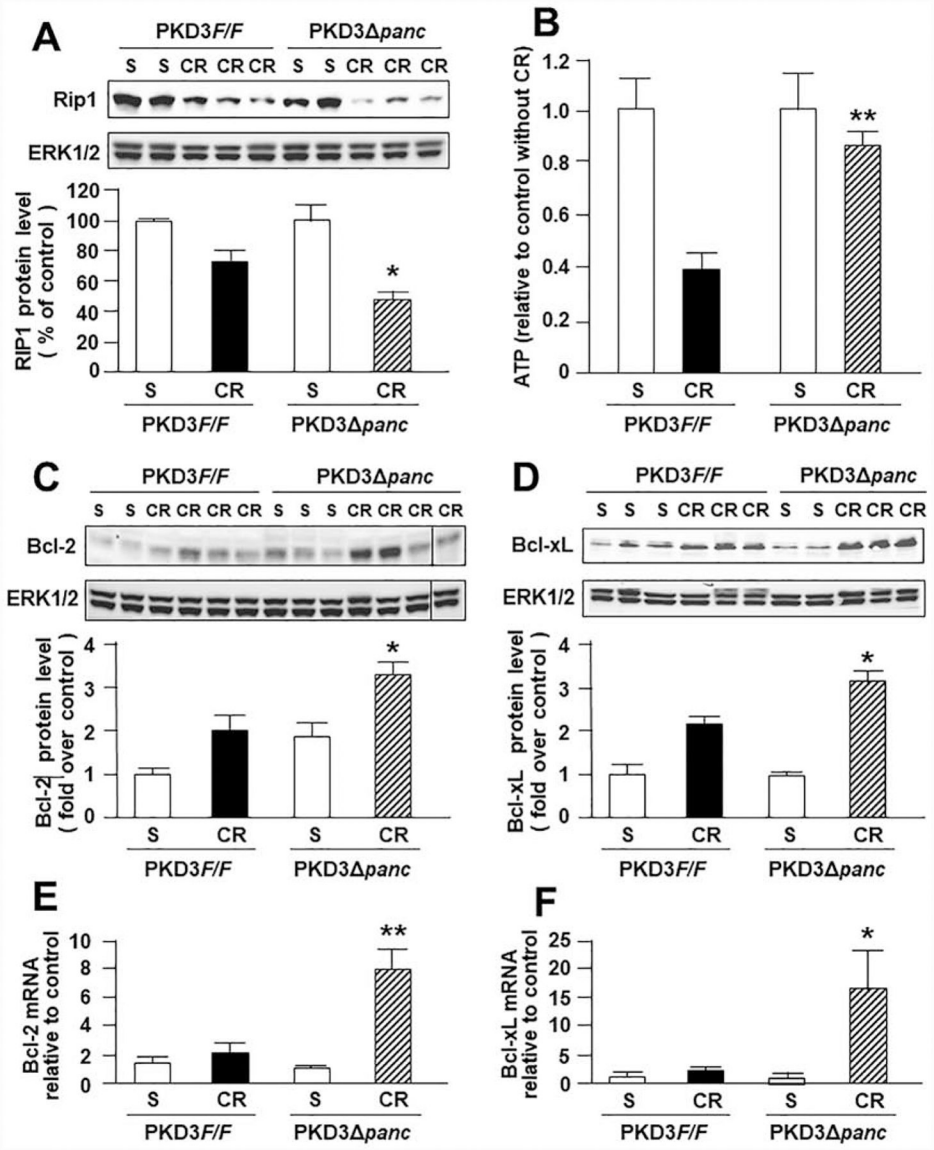

Fig. 9.

Pancreas-specific deletion of PKD3 promoted RIP1 degradation/inactivation, prevented ATP depletion, and upregulated pancreatic prosurvival Bcl-2 family protein and mRNA expression in cerulein-induced pancreatitis. Western blot analyses of pancreatic tissue lysate were performed using antibodies against RIP1 (A), Bcl-2 (C) and Bcl-xL (D) to measure their protein levels in cerulein (CR) pancreatitis. Blots were also probed for ERK1/2 to confirm equal protein loading. The bar figures (in A, C, D) are the quantification of these protein band densities that are normalized to ERK1/2 and then compared to saline (S) control. Values are means ± SEM for each condition. Each lane represents an individual mouse. n=2–3 mice per strain for saline treatment, n=3–5 mice per strain for CR treatment. $*P < 0.05$ or $**P < 0.01$ versus PKD3*F/F* mice with CR (black bars). Samples were run in a single gel but were not continuous, as indicated by a line between lanes (C). (B) Pancreatic ATP level was determined and the ATP values in PKD3*F/F* or PKD3*Δpanc* mice were

compared to their own control mice treated with saline. Values are means ± SEM (n=3). * *P* < 0.01 versus PKD3*F/F* mice injected with cerulean (CR, black bar). (E, F) mRNA extracts from pancreas of PKD3*F/F* and PKD3*Δpanc* mice were analyzed by qPCR to determine the expression of Bcl-2 (E) and Bcl-xL (F). Bar Values are means ± SEM (n>5), * *P* < 0.05 or * * *P* < 0.01 versus PKD3*F/F* control mice with CR.

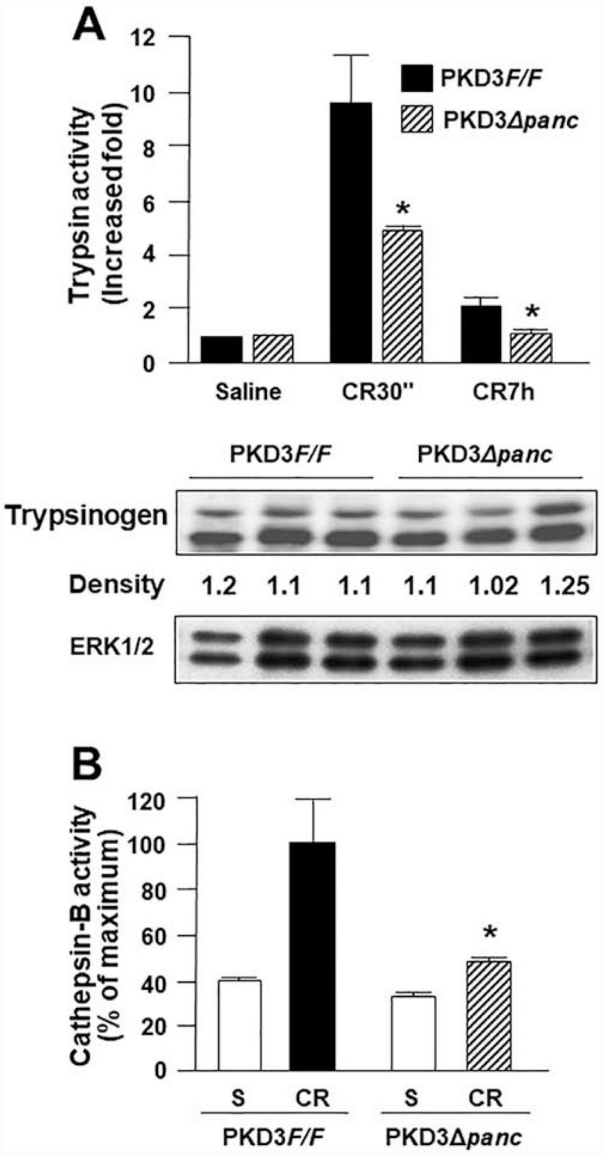

Fig. 10.

Pancreas-specific PKD3 deletion decreased intrapancreatic trypsinogen activation and cathepsin B activation in cerulein pancreatitis. (A) Pancreatic trypsin activities at indicated times of pancreatitis were measured. Values are means ± SE (n>4), **P* < 0.05 versus PKD3*F/F* mice treated with cerulein. Western blot analysis of pancreatic trypsinogen (*Lower panel*) shows that pancreas specific PKD3 deletion does not alter pancreatic expression of this enzyme; Blots of ERK1/2: for loading controls; The trypsinogen protein band density was quantified and normalized to the loading control ERK. Each lane represents an individual mouse, n=3. (B) Pancreatic cathepsin B activities were measured. Values are means ± SME (n>5), **P* < 0.05 versus PKD3*F/F* mice treated with cerulein.

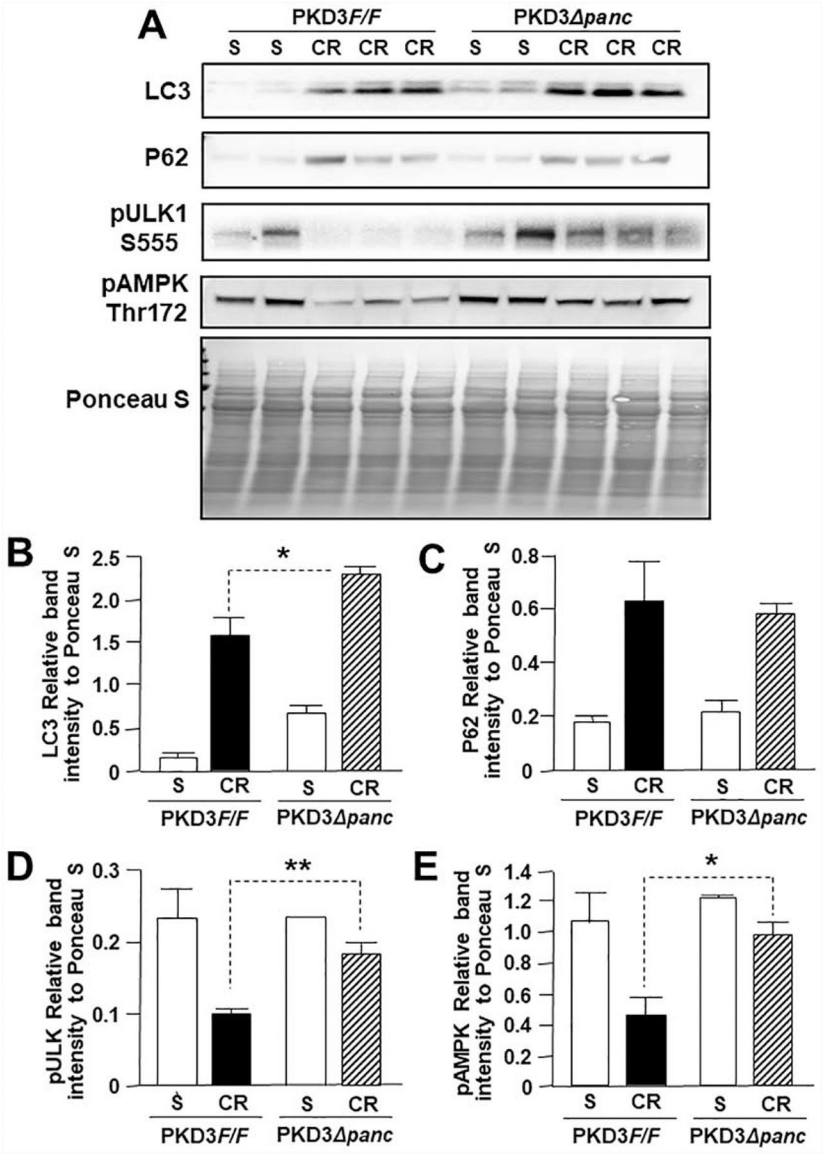

Fig. 11.

Pancreas-specific PKD3 deletion enhances autophagy flux. (A) Western blot showing the expression of LC3, p62, pULK1 (S555), and pAMPK (T172) in post nuclear supernatant. (B-E) Bar figures represent the expression of these proteins normalized to Ponceau S stain. Values in graphs are represented as means ± SME. Each lane represents an individual mouse. n=2 for saline treatment, n=3 for CR treatment. $^{**}P < 0.01$ or $^{*}P < 0.05$ vs PKD3*F/F* mice with cerulein.

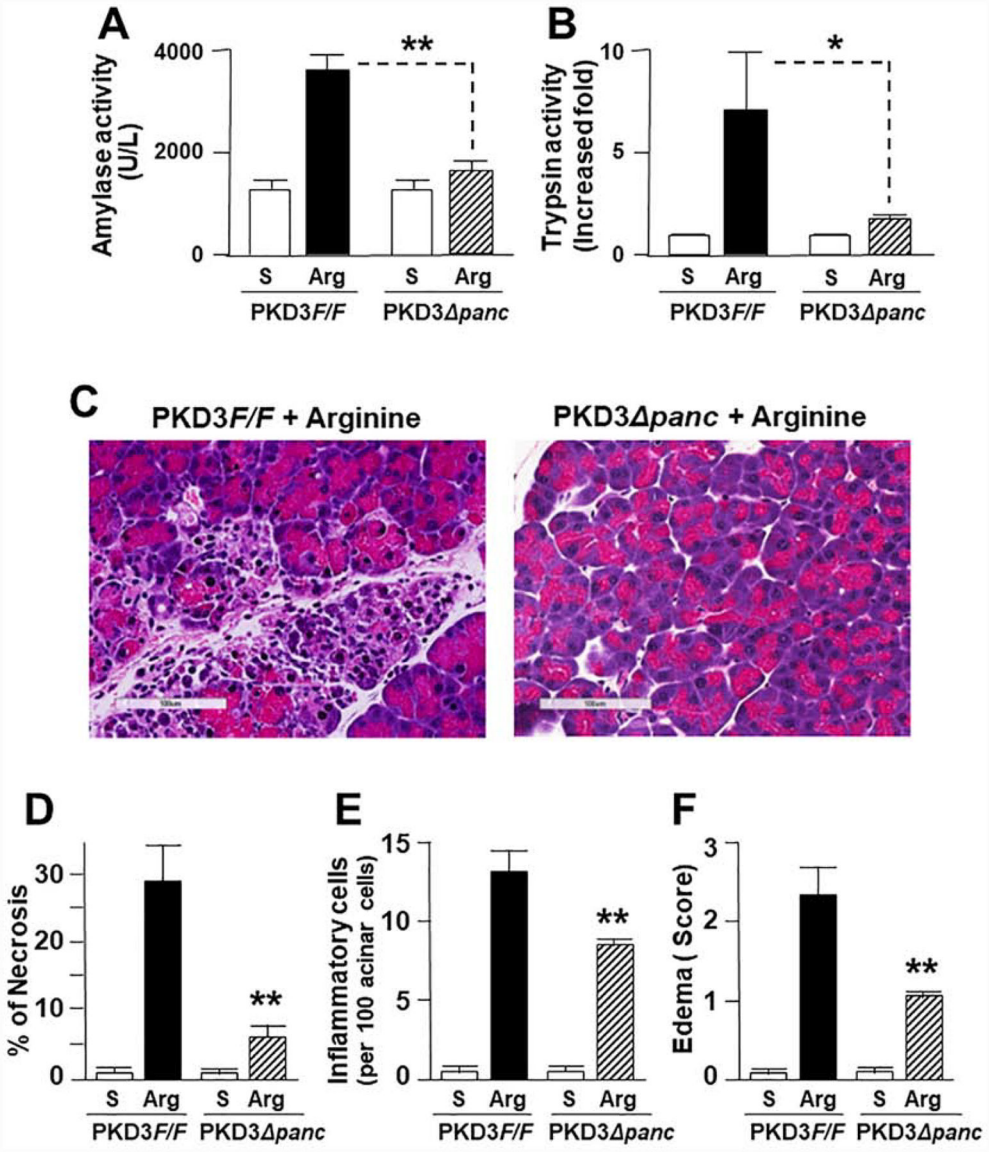

Fig. 12.

Pancreas-specific PKD3 deletion attenuated inflammation, necrosis and severity in L-arginine-induced pancreatitis. Pancreas-specific PKD3 deletion decreased blood amylase (A) and pancreatic trypsinogen activation (B) in arginine pancreatitis. Values are means ± SEM (n>6), $^{**}P < 0.01$ or $^{*}P < 0.05$ versus PKD3*F/F* control mice with arginine. (C) H&E staining of pancreatic tissue sections from mice with L-arginine injections. Each image was representative of at least 5 mice with similar results at each condition. Bars represent 100μm. Necrosis (D) and inflammatory cells infiltration (E) were measured on H&E stained pancreatic tissue sections, as described in Methods, and expressed as percentage of total acinar cells. (F) Pancreatic edema grading was made from 1 to 3 on the H&E stained tissue sections with Schoenberg grading system as described in Fig. 3. Results in graphs D-F represent means ± SEM (n=5 mice) for each condition. $^{**}P < 0.01$ versus PKD3*F/F* with arginine injections as indicated.

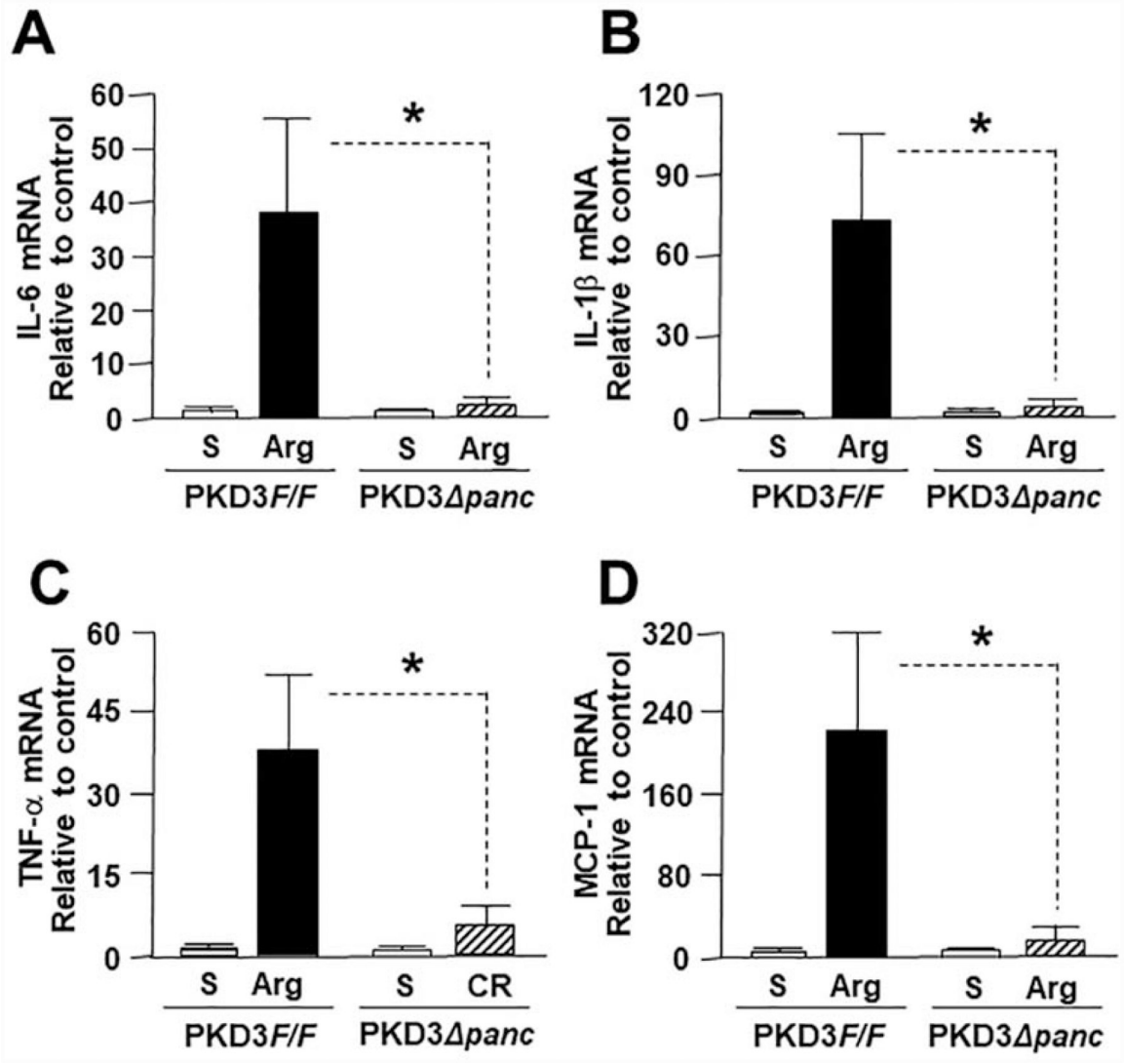

Fig. 13.

Pancreas-specific deletion of PKD3 decreased mRNA expression of inflammatory molecules in arginine pancreatitis. mRNA extracts from pancreas of PKD3*F/F* and PKD3*Δpanc* mice were analyzed by real-time quantitative PCR to determine the expression of IL-6 (A), IL-1β (B), TNF-α (C), and MCP-1 (D) in L-arginine (Arg)-induced pancreatitis. Bar values are means ± SEM (n>5), **P* < 0.05 versus PKD3*F/F* control mice with Arg.

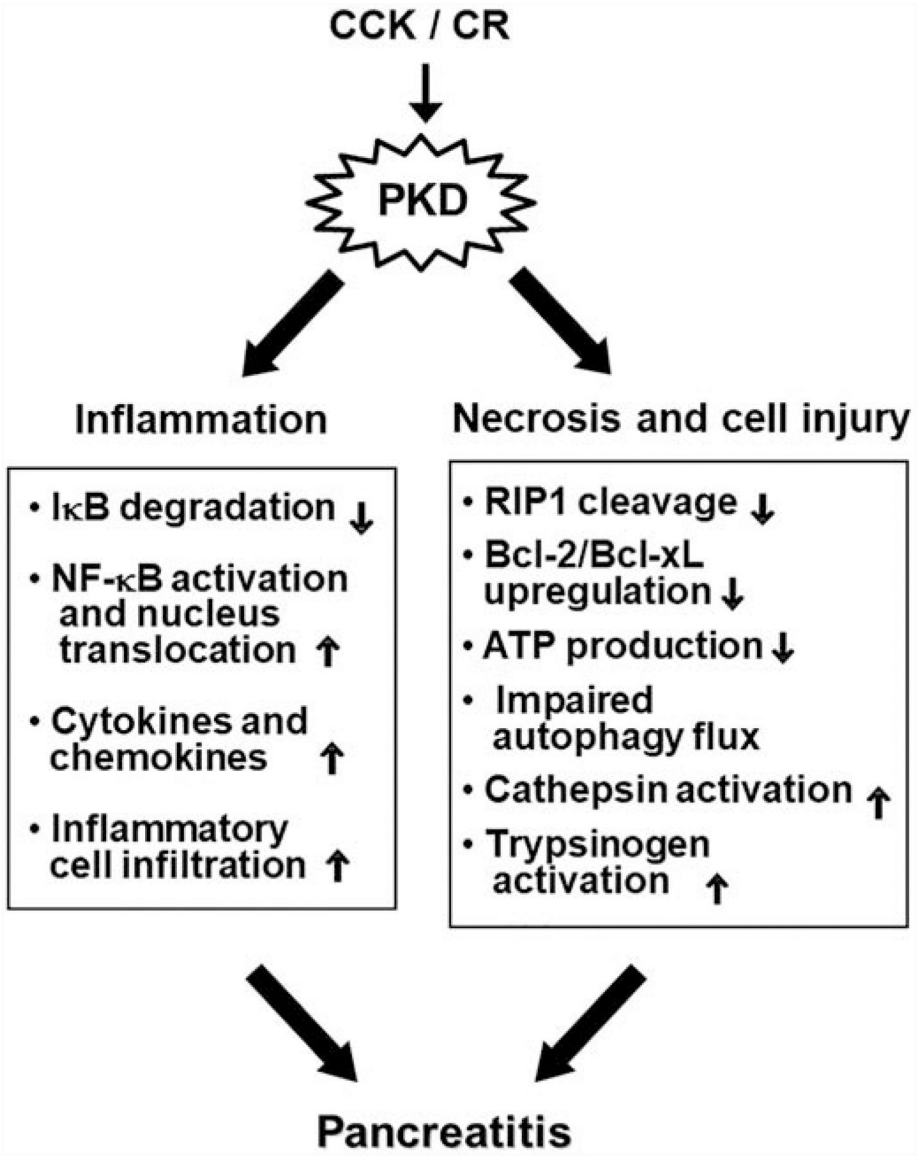

Fig. 14.

A scheme illustrating the pathways/mechanisms of PKD3 mediating pathology of pancreatitis through regulation of inflammation, necrosis, and cell injury.

Table 1.

Primer Sequences for Real-Time Quantitative PCR

Transcript	Forward primer	Reverse primer
Mouse MCP-1	5’-ATGCAGTTAACGCCCCACTC	5’- TTCTTTGGGACACCTGCTGC
Mouse IL-6	5’-CGTGGAAATGAGAAAAGAGTTGTG	5’-CCAGTTTGGTAGCATCCATCATTTCT
Mouse IL-1β	5’-GCAACTGTTCCTGAACTCAACT	5’- ATCTTTTGGGGTCCGTCAACT
Mouse TNF-α	5’-CATCTTCTCAAAATTCGAGTGACAA	5’- T GGGAGT AGACAAGGT ACAACCC
Mouse Bcl-2	5’-GACTGAGTACCTGAACCGGC	5’- AGTTCCACAAAGGCATCCCAG
Mouse Bcl-xL	5’- GGTCTCTTCAGGGGAAACTGAG	5’- GCCAAGATAAGGTTCTGGCTGA
Mouse 18S	5’- AGTCCCTGCCCTTTGTACACA	5’- CGATCCGAGGGCCTCACTA

Note: 18S, 18S ribosomal RNA