CELL INJURY, REPAIR, AGING, AND APOPTOSIS

Dichotomy between Receptor-Interacting Protein 1— and Receptor-Interacting Protein 3—Mediated Necroptosis in Experimental Pancreatitis

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Pancreatic acinar cell necrosis and inflammatory responses are two key pathologic processes in acute pancreatitis (AP), which determines the severity and outcome of the disease. Recent studies suggest that necroptosis, a programmed form of necrosis, is involved in the pathogenesis of AP, but the underlying mechanisms remain unknown. We investigated the expression of necrosome components, including receptor-interacting protein (RIP) 1, RIP3, and mixed lineage kinase domain-like (MLKL), and the molecular mechanisms in pancreatitis-associated necroptosis. We found that RIP3 and phosphorylated MLKL expression was positively related to the degree of necrosis, whereas RIP1 expression was negatively related to the degree of necrosis. Pharmacologic inhibition of RIP1 kinase activity exerted no protection against caerulein/cholecystokinin-8–induced AP, but knockdown of RIP1 with siRNA increased acinar cell necrosis and inhibition of NF- κ B activation. RIP1 inhibition led to enhanced RIP3 expression. RIP3 and MLKL inhibition decreased acinar cell necrosis, in which the inhibition of RIP3 reduced the phosphorylation level of MLKL. RIP3 inhibition had no effect on trypsinogen activation but partly inhibited inflammasome activation. Our study strongly suggests that the imbalance between RIP1 and RIP3 shifts the cell death to necrosis, which unravels a new molecular pathogenesis of mechanism of AP and may provide insight into the development of novel therapeutic agent for other necrosis-related diseases. (*Am J Pathol* 2017, 187: 1035–1048; <http://dx.doi.org/10.1016/j.ajpath.2016.12.021>)

Acute pancreatitis (AP) is a cell necrosis–related disease. To date, no specific therapy has been found to be effective for AP, which has a high morbidity and mortality,^{1,2} because the exact mechanism(s) involved remains unclear. For most patients, AP is mild and resolves spontaneously; however, in severe cases, the inflammation intensifies and presents as severe AP (SAP).

There are two main forms of cell death in pancreatic acinar cells, including necrosis and apoptosis. Acinar cell necrosis plays a pivotal role in SAP.³ The extent of acinar cell necrosis correlates with the severity of AP.^{4–6} On the contrary, induction of acinar apoptosis protects from pancreatic damage.⁷ Necrosis has long been described as an accidental and uncontrolled event. However, many recent studies revealed that many cellular stimuli, including tumor necrosis factor (TNF)- α , CD95, and TNF-related

apoptosis-inducing ligand, can induce a programmed necrotic process independent of caspases, named necroptosis.^{8,9} Necroptosis plays a central role in a number of disease models and pathologic conditions, including ethanol-induced liver injury,¹⁰ ischemia or reperfusion injury,¹¹ viral infection,¹² ileitis,¹³ and AP.^{14,15} The capability of cells to undergo necroptosis depends on the absence of caspases and interaction of receptor interacting protein (RIP) 1 and RIP3 to form necrosome.¹⁶ RIP homotypic interaction motif is a key structure domain in mediating the

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formation of the RIP1/RIP3 complex and further recruiting other components, such as mixed lineage kinase domain-like (MLKL).¹⁷ RIP3 is an indispensable component in regulating the conversion of TNF-induced apoptosis to necroptosis. Genetic depletion of RIP3 reduced renal ischemic reperfusion injury,¹⁸ cardiac hypoxic injury,¹⁹ and brain injury.²⁰ RIP1 plays a crucial role in regulating phosphorylation of RIP3 and subsequently initiating necroptotic signaling.¹² Degterev and colleagues²¹ discovered necrostatin (Nec)-1 as a specific inhibitor of necroptosis through inhibiting kinase activity of RIP1. Nec-1 has been reported to rescue TNF-stimulated cells from necroptosis and also to reduce multiorgan damage in various disease models. Meanwhile, RIP1 is also a key mediator of TNF-induced canonical NF- κ B signaling, indicating a dual role of RIP1 in regulating cell death and survival.⁹ In some conditions, RIP3 was proved to induce necroptosis independently in the absence of RIP1. Elevation of RIP3 allows TNF to directly activate necroptotic signaling in mouse but not rely on RIP1.²² The absence of RIP3 but not treatment with Nec-1 prevented ethanol-induced liver damage.¹⁰ In another study, Nec-1 and another necroptosis inhibitor TAT-crmA even exacerbated TNF/zVAD-induced shock and accelerated time to death.²³

He et al¹⁴ and Zhang et al¹⁵ reported that genetic depletion of RIP3 significantly ameliorated AP induced by caerulein in C57/B6 mice. Interestingly, pancreatic damage induced by caerulein was not protected in the presence of Nec-1.²³ These results indicated that RIP1 may have complex biological functions in AP. To elucidate the exact role of RIP1 and RIP3 and their interaction relationship in AP, we established different severity of the AP model *in vivo* and *in vitro*. In the present study, we found that the imbalance between RIP1 and RIP3 shifts the cell death to necrosis in AP, which unravels a new molecular pathogenesis of mechanism of SAP and may provide insight into the development of novel therapeutic agent for other necrosis-related diseases.

Materials and Methods

Reagents

L-Arginine monohydrochloride (L-Arg) (A5131), caerulein (C9026), cholecystokinin-8 (CCK) (C2175), Bay 11-7085 (B5681), and propidium iodide (PI) (P4170) were purchased from Sigma-Aldrich (St. Louis, MO). Nec-1 (480065), Nec-1 inactive control (Nec-1i) (480066), and zVAD (#219007) were purchased from Calbiochem (Merck Millipore, Vienna, Austria). Hoechst 33342 (C1026) was purchased from Beyotime Biotechnology (Jiangsu, China). Antibody against RIP1 (610458) was purchased from BD Biosciences (San Jose, CA); RIP3 (sc-135171), caspase-1 (sc-56036), and β -actin (sc-81178) were purchased from Santa Cruz Biotechnology (Dallas, TX); NF- κ B (ab16502), MLKL (ab172868), phosphorylated MLKL (pMLKL) (ab196436),

and histone H3 (ab8580) were purchased from Abcam (Cambridge, UK); Ly-6G (104702) was from BioLegend (San Diego, CA); IL-1 β (3A6), NLRP3 (15101), caspase-3 (9665), caspase-8 (4790), and caspase-9 (9508) were purchased from Cell Signaling Technology (Danvers, MA).

Induction of Experimental Pancreatitis

Male C57BL/6, BALB/c, Institute of Cancer Research (ICR) mice, and Sprague-Dawley (SD) rats at 7 to 8 weeks of age were purchased from Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China). All experiments were conducted with the approval of the Animal Research Committee at Shanghai Jiaotong University. Caerulein pancreatitis was induced in mice and rats by 7 or 10 i.p. injections of 50 μ g/kg of caerulein once every hour, as previously described.²⁴ Control animals received similar injections of physiologic saline. Animals were sacrificed at several intervals from 0 to 12 hours after the first i.p. injection of caerulein, and samples of tissues and blood were rapidly harvested.

Isolation of Pancreatic Acinar Cells

Pancreatic acinar cells were isolated from mice using a collagenase digestion procedure as previously described.²⁴ For treatments, the isolated acinar cells were incubated at 37°C in Dulbecco's modified Eagle's medium/Ham F-12 medium containing 20% fetal bovine serum with or without CCK or L-Arg and other agents, as described in the relevant figures.

siRNA Transfection

For siRNA transfections, pancreatic acinar cells were transfected with 150 nmol/L siRNA oligonucleotides using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer protocol. Transfected cells were tested for a response to CCK or other agents 24 hours after transfection. Efficacy of knocking down was performed by Western blotting analysis. The siRNA sequence used for transfections is given in Table 1.

Serum Amylase and Lipase Assay

The serum activities of amylase and lipase were measured by enzyme dynamics chemistry using commercial kits according to the manufacturer's protocols in a Roche/Hitachi modular analytics system (Roche, Berlin, Germany).

Histologic Examination of the Pancreas and Lung

Fresh specimens of animal pancreas and lung were fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). Tissues were embedded in paraffin, and 5- μ m sections were processed for hematoxylin and eosin (H&E) staining using standard procedures.

Table 1 siRNA Oligo-Nucleotides Sequences

Gene (mouse)	Oligo-nucleotides sequences
<i>Ripk1</i> (alias <i>Rip1</i>) #1	
Sense	5'-GAGGAUAUUCUCAGGCUUCAGGUCCUU-3'
Antisense	5'-GGACCUGAAGCCUGAGAAUAUCCTC-3'
<i>Ripk1</i> (alias <i>Rip1</i>) #2	
Sense	5'-CCUUCGUUCCUUUCCUCCUCUCUGUU-3'
Antisense	5'-CAGAGAGGAGGAAAGGAAACGAAGG-3'
<i>Ripk1</i> (alias <i>Rip1</i>) #3	
Sense	5'-CCACUAGUCUGACUGAUGATT-3'
Antisense	5'-UCAUCAGUCAGACUAGUGGTT-3'
<i>Ripk1</i> (alias <i>Rip1</i>) #4	
Sense	5'-GAAUGAGGCUUACAACAGTT-3'
Antisense	5'-CUGUUGUAAGCCUCAUUCTT-3'
<i>Ripk3</i> (alias <i>Rip3</i>) #1	
Sense	5'-CGACGAUGUCUUCUGUCAATT-3'
Antisense	5'-UUGACAGAAGACAUCGUCGTT-3'
<i>Ripk3</i> (alias <i>Rip3</i>) #2	
Sense	5'-AGCUGUUAUUUAAAGUCAATT-3'
Antisense	5'-UUGACUUUAAAUACAGCUTT-3'
<i>Ripk3</i> (alias <i>Rip3</i>) #3	
Sense	5'-CCUUAAGUCAUAAACAUTT-3'
Antisense	5'-AUGUUUAUUGACUUUAGGTT-3'
<i>Ripk3</i> (alias <i>Rip3</i>) #4	
Sense	5'-GGGAAACCAUGGUUUCUAATT-3'
Antisense	5'-UUAGAAACCAUGGUUCCCTT-3'
<i>Mkl1</i> #1	
Sense	5'-GAGAUCCAGUUAACGAUATT-3'
Antisense	5'-UAUCGUUGAACUGGAUCUCTT-3'
<i>Mkl1</i> #2	
Sense	5'-GGAAUACCGUUUCAGAUGUTT-3'
Antisense	5'-ACAUCUGAAACGGUAUUCCTT-3'

Assessment of Apoptosis in the Pancreas

Apoptosis was quantified on pancreatic tissue samples by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay. Briefly, pancreas tissues were fixed in 4% buffered formaldehyde and embedded in paraffin, and 5- μ m sections were stained using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay according to the manufacturer's protocol (Roche). The numbers of apoptotic cells were counted in 10 randomly chosen microscopic fields ($\times 200$) as described previously.²⁴

Assessment of Necrosis in the Pancreas

Necrosis in pancreatic tissue was quantified on sections stained with H&E. The extent of acinar cell injury or necrosis was scored by two experienced morphologists (X.H. and B.L.), as previously described.^{5,25} Ten randomly chosen microscopic fields ($\times 200$) were examined for each sample, and the extent of acinar cell necrosis was expressed as a percentage of total acinar cells. Cells with swollen cytoplasm, loss of plasma membrane integrity, and leakage of organelles into the interstitium were considered necrotic.

Necrosis in pancreatic acinar cells was determined using Hoechst 33342 and PI staining.²⁴ Briefly, cells were stained with 8 mg/mL of Hoechst 33342 and 1 mg/mL of PI and were examined directly by fluorescence microscopy. Ten randomly chosen microscopic fields ($\times 200$) were examined for each sample, and the extent of acinar cell necrosis was expressed as a percentage of total acinar cells. Cells with swollen cytoplasm, loss of plasma membrane integrity, and stained by PI were considered necrotic.

Immunohistochemistry

Mice lung tissues were fixed in 4% buffered formaldehyde and embedded in paraffin, and 5- μ m sections were adhered to glass slides. Ly-6G antibody (1:50) was used for immunohistochemistry according to established procedures. Briefly, after deparaffinization and antigen retrieval, nonspecific bindings were blocked and primary antibodies were applied (4°C, overnight). After washing, incubation with the appropriate horseradish peroxidase-labeled polymers (BioCare, Kloten, Switzerland) was performed. Positive labeling was detected by exposing the sample to substrate 3,3'-diaminobenzidine (Phoenix Biotechnologies, San Antonio, TX). Samples were then counterstained with hematoxylin solution.

ATP Analysis

Cell survival assay was performed using the Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI). A Cell Titer-Glo Assay was performed according to the manufacturer's instructions. Briefly, an approximately 100-mL cell suspension was spotted onto a 96-well culture plate. ATP detection reagents were added, and the level of bioluminescence was detected.

Western Blotting

Western blotting analysis was performed on pancreatic tissue, pancreatic whole cell extracts, or nuclear extracts. Total amounts of protein were detected using the bicinchoninic acid assay method (Beyotime Biotechnology, China). Proteins were submitted to a sodium dodecyl sulfate (SDS)-polyacrylamide gel, and blotted with standard procedures. Five percent bovine serum albumin (BSA) in tris-buffered saline (TBS) was used to block non-specific binding. The membranes were then incubated with a primary antibody overnight at 4°C. Next, membranes were washed with TBS containing 0.1% Triton and incubated with the secondary antibody horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:2000) for 1 hour at room temperature. After washing with tris-buffered saline with Tween 20 (TBST), membranes were developed and exposed to chemiluminescence (ECL) film (Santa Cruz). Finally, the membranes were washed three times and scanned using the Odyssey two-color

infrared laser imaging system (fluorescence detection). Representative blots were presented from three independent experiments. Quantitative densitometric analyses of immunoblotting images were performed using ImageJ software version 1.44p (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>). The relative expression of proteins was expressed as fold changes compared to the control in the blot after normalized to β -actin or the total proteins in some experiments.

Immunoprecipitation

Primary pancreatic acinar cells were isolated and cultured on 10 cm dishes. Cells were stimulated with 200 nmol/L CCK for six hours and harvested after washing twice with phosphate-buffered saline. The harvested cells were lysed for 30 minutes on ice in the lysis buffer and then spun down at 12,000 g for 20 minutes. The soluble fraction was collected, and the protein concentration was determined by bicinchoninic acid assay. Next, 1 mg of extracted protein in lysis buffer was immunoprecipitated overnight with anti-RIP3 affinity gel (Sigma-Aldrich) at 4°C. The immunoprecipitates were washed three times with lysis buffer. The beads were then eluted with 0.5 mg/mL of the corresponding antigenic peptide for 4 hours or directly boiled in 1% SDS loading buffer.

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared with nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL). A single-stranded 30-mer biotin-labeled probe containing the NF- κ B consensus site was purchased from Beyotime Biotechnology (Beyotime Biotechnology). The electrophoretic mobility shift assay binding reactions were performed using a Light Shift chemiluminescent electrophoretic mobility shift assay kit (Pierce). Specifically, 5 μ g of nuclear extract was incubated in 1 $\times$ binding buffer containing 2.5% glycerol, 0.05% NP-40, 50 mmol/L KCl, 5 mmol/L MgCl₂, 50 ng poly(dI-dC), and biotinylated probe with or without protein extract for 30 minutes at room temperature. The complexes were separated on a 5% polyacrylamide-0.5 $\times$ Tris-borate-EDTA gel and transferred to a positively charged nylon membrane. After the transfer was completed, the membrane was crosslinked and biotin-labeled DNA was detected using a chemiluminescent detection kit (Pierce).

Enzyme-Linked Immunosorbent Assay

Whole cell extracts of pancreatic acinar cells were harvested and stored at -80°C for further detection. The concentrations of cleaved IL-1 β (Abcam) and trypsinogen activation peptide (TAP) (Xinqidi Biological Technology Co., Ltd, Wuhan, China) in pancreatic acinar cells and proinflammatory cytokines (TNF- α , IL-6, and IL-1 β) (Shanghai Westang Bio-Tech Co. Ltd., Shanghai, China) in cell culture

Table 2 PCR Genes Primers Sequences and Annealing Temperature

Gene (mouse)	Primer sequences	Annealing temperature
<i>Tnfα</i>		
Forward	5'-CCTCACACTCACAAACCACCA-3'	62°C
Reverse	5'-ACAAGGTACAACCCATCGGC-3'	
<i>Il6</i>		
Forward	5'-CCGGAGAGGAGACTTCACAG-3'	62°C
Reverse	5'-CATTTCCACGATTTCCAGA-3'	
<i>Il1β</i>		
Forward	5'-ATGCCACCTTTTGACAGTGATG-3'	62°C
Reverse	5'-TGATGTGCTGCTGCCGAGATT-3'	
<i>Actb</i>		
Forward	5'-ACCTTCTACAATGAGCTGCG-3'	56°C
Reverse	5'-CTGGATGGCTACGTACATGG-3'	

supernatants were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocols (Abcam; Xinqidi Biological Technology Co., Ltd; and Shanghai Westang Bio-Tech Co. Ltd.). The cleaved IL-1 β and TAP protein levels were expressed in nanograms per gram of tissue.

RT-PCR

Total RNA was extracted from mice pancreas following the acid guanidinium, phenol, and chloroform method in the presence of liquid nitrogen. Reverse transcription was performed using the commercial SuperScript II pre-amplification kit (Fermentas, Glen Burnie, MD). The synthesized cDNA was then subjected to RT-PCR. All the procedures were performed using ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The mRNA expression levels of target genes, including TNF- α , IL-6, and IL-1 β , were normalized to the housekeeping gene β -actin. Each target gene was analyzed in triplicate under each triplicate experiment. Fold changes of gene expression were then calculated and expressed as relative transcript levels ($2^{\Delta\Delta}$). The primer sequences are listed in Table 2.

Statistical Analysis

Data are presented as means $\pm$ SEM, representing replicates within an experiment. Statistical significance was determined by the *t*-test. $P < 0.05$ was considered statistically significant.

Results

Necrosis/Apoptosis Ratio Determines the Severity of Experimental Pancreatitis

After repetitive administration of caerulein in C57BL/6 mice and SD rats, H&E staining revealed that pathologic alterations of pancreatic morphologic findings, including

Figure 1 Morphologic characterization and cell death type in different caerulein-induced experimental acute pancreatitis (AP) models. **A–E:** Sprague-Dawley rats and C57BL/6 mice were given i.p. injections of 50 µg/kg of caerulein or saline once every hour for 7 hours. At 0, 3, 6, 9, and 12 hours after the first injection, mice and rats were sacrificed, and the samples of tissues were harvested. **A:** The pancreatic tissue was stained with hematoxylin and eosin (H&E). **Arrows** indicate necrotic acinar cells. **B:** Pancreatic sections were analyzed using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). **Arrows** indicate apoptotic acinar cells. **C:** Percentage of necrotic cells was determined as described in the *Materials and Methods*. **D:** Number of apoptotic cells in 10 fields. **E:** The proteins were extracted from indicated tissues and subjected to Western blotting analysis of caspase-3, caspase-8, caspase-9, and β-actin levels. **F and G:** C57BL/6, BALB/c, and Institute of Cancer Research (ICR) mice were given i.p. injections of 50 µg/kg of cerulein or saline once every hour for 7 hours. Three hours after the last injection, mice were sacrificed, and the samples of tissues were harvested. **F:** The pancreatic tissue was stained with H&E. **Arrows** indicate necrotic acinar cells. **G:** Percentage of necrotic cells was determined as described in the *Materials and Methods*. **H:** Primary pancreatic acinar cells were isolated from C57BL/6, BALB/c, and ICR mice and treated with 200 nmol/L cholecystokinin-8 for 6 hours. Cell viability was determined by measuring ATP levels. All experiments were performed at least three times. Data are presented as means ± SEM. **P* < 0.05 versus rat; †*P* < 0.05 versus C57BL/6; ‡*P* < 0.05 versus BALB/c. Scale bars: 50 µm (**A** and **B**); 100 µm (**F**). Original magnification: ×200 (**D**). Cle-, cleaved; NC, negative control.

Figure 2 Protein expression of necrosome components in pancreatic tissue or pancreatic acinar cells after caerulein/cholecystokinin-8 (CCK) stimulation. **A:** C57BL/6, BALB/c, and Institute of Cancer Research (ICR) mice were given i.p. injections of 50 µg/kg of caerulein or saline once every hour for 7 hours. Three hours after the last injection, mice were sacrificed and the samples of tissues were harvested. The proteins were extracted from indicated tissues and subjected to Western blotting analysis of receptor-interacting protein (RIP) 1, RIP3, and β-actin. Representative blots from three independent experiments and band intensities determined by densitometry scanning were presented. The relative protein expression of RIP1 and RIP3 was expressed as fold changes compared with the control in the blot after normalized to β-actin. **B:** Primary pancreatic acinar cells were isolated from C57BL/6, BALB/c, and ICR mice and were treated with 200 nmol/L CCK for 6 hours. Whole cell extracts were subjected to Western blotting analysis of RIP1 and RIP3 and β-actin by Western blotting analysis. Representative blots from three independent experiments and band intensities determined by densitometry scanning were presented. The relative protein expression of RIP1 and RIP3 was expressed as fold changes compared with the control in the blot after normalized to β-actin. **C:** The extent of RIP1 up-regulation correlated inversely with acinar cell necrosis in pancreas tissue after caerulein stimulation (*in vivo*) and isolated pancreatic acinar cells after CCK stimulation (*in vitro*). The solid column indicated the percentage of necrotic cells, and the hollow column corresponds to the relative protein expression of RIP1. **D:** The extent of RIP3 up-regulation correlated positively with acinar cell necrosis in pancreas tissue after caerulein stimulation (*in vivo*) and isolated pancreatic acinar cells after CCK stimulation (*in vitro*). The solid column indicates the percentage of necrotic cells, and the hollow column corresponds to the relative protein expression of RIP3. All experiments were performed at least three times. Data are represented as means ± SEM. **P* < 0.05 versus NC. AP, acute pancreatitis; NC, negative control.

edema, acinar cell necrosis, and inflammatory cell infiltration, were much more pronounced in mice than in rats (Figure 1A). Caerulein-treated mice had multiple areas of pancreatic acinar cell loss and necrosis, whereas caerulein-treated rats had scarce necrosis. Time-dependent changes in the extent of necrosis and apoptosis in the pancreas were measured using H&E staining and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining, respectively (Figure 1, A–D). The degree of pancreas necrosis was elevated after caerulein injection in both rat and mouse models in a time-dependent manner and peaked necrosis at 12 hours (Figure 1C). At each time point, the extent of necrosis in the mouse model was more severe than that in the rat model. The extent of apoptosis in both

mice and rats reached a maximum at 9 hours and decreased at 12 hours, but the number of apoptotic cells in rats was much greater than in mice (Figure 1D). Western blotting analysis also revealed apoptosis to be predominant in rats but weak in mice (Figure 1E).

To further confirm the role of necrosis in determining AP severity, we developed caerulein/CCK-induced experimental AP models in three different species of mice: C57BL/6, BALB/c, and ICR mice. At 9 hours after caerulein injection, pancreatic damage was observed to be most severe in ICR mice, moderate in BALB/c mice, and mild in C57BL/6 mice (Figure 1, F and G). The extent of lung injuries and the degree of neutrophil infiltration were also most severe in ICR mice, moderate in BALB/c mice, and

Figure 3 The receptor-interacting protein (RIP) 1 kinase inhibitor necrostatin (Nec)-1 exerts no protective effect against cerulein-induced mice acute pancreatitis (AP) *in vivo*. **A–D:** C57BL/6 mice were given i.p. injections of 50 μ g/kg of cerulein or saline once every hour for 10 hours. RIP1 kinase inhibitor Nec-1 or Nec-1 inactive control (Nec-1i) dissolved in 4% dimethyl sulfoxide were administered i.p. every 6 hours, injected 2 hours before cerulein injection. The dosage of Nec-1 was 0.5 mg/kg (the Nec-1L group), 1 mg/kg (the Nec-1M group), and 2 mg/kg (the Nec-1H group). The dosage of Nec-1i was 1 mg/kg. Then 12, 18, and 24 hours after the first injection, mice were sacrificed, and the samples of tissues and blood were harvested. **A:** Hematoxylin and eosin staining of mouse pancreas in cerulein-injected mice with or without Nec-1 or Nec-1i. Serum amylase (**B**) and lipase (**C**) levels and necrosis score (**D**) in cerulein-injected mice with or without Nec-1 or Nec-1i. All experiments were performed at least three times. Data are presented as means $\pm$ SEM. Scale bar = 100 μ m (**A**).

mild in C57BL/6 mice (Supplemental Figure S1, A and B). To determine whether differences in the extent of necrosis found in the different species of mice models occur at the level of acinar cells, we compared the effects of CCK to the necrosis in acinar cells isolated from mice pancreas. Stimulation of pancreatic acinar cells with CCK at 200 nmol/L for 6 hours revealed that intracellular ATP depletion was $43.84\% \pm 10.41\%$ in ICR mice, $29.13\% \pm 5.20\%$ in BALB/c mice, and $16.04\% \pm 7.53\%$ in C57BL/6 mice (Figure 1H). These results also indicate that the necrosis differences in mouse cerulein pancreatitis occur in acinar cells.

Expression of RIP1 and RIP3 in AP

To investigate the molecular mechanism of necroptosis in AP, we detected protein expression of RIP1 and RIP3 in cerulein/CCK-induced experimental AP models or pancreatic acinar cells in three different species of mice. As

expected, the RIP3 protein level was up-regulated in mouse pancreatitis tissue and CCK-stimulated pancreatic acinar cells (Figure 2, A and B). In addition, the expression was mostly elevated in ICR mouse pancreatitis tissue, moderately elevated in BALB/c mice, and mildly elevated in C57BL/6 mice. A significant reduction of RIP1 expression was observed in ICR mice, although the RIP1 protein level was increased in C57BL/6 and BALB/c mice (Figure 2, A and B). The expression of RIP1 in different species revealed an inverse relationship with the degree of necrosis (Figure 2C), whereas the expression of RIP3 was consistent with the degree of necrosis and severity of AP (Figure 2D).

RIP1 Kinase Inhibitor Exerts No Protective Effect against Experimental Pancreatitis *in Vivo* and *in Vitro*

To further investigate the role of RIP1 in pancreatic acinar cell necrosis, the RIP1 kinase inhibitor Nec-1 or Nec-1i

Figure 4 Receptor-interacting protein (RIP) 1 kinase inhibitor exerts no protective effect against cholecystokinin-8 (CCK)/L-Arginine monohydrochloride (L-Arg)-induced mice pancreatic acinar cell necrosis *in vitro*. **A** and **B**: Primary pancreatic acinar cells were isolated from C57BL/6 mice and were treated with CCK, L-Arg, necrostatin-1 (Nec-1), Nec-1 inactive control (Nec-1i), and zVAD for 6 hours. Cell viability was determined by measuring ATP levels. All experiments were performed at least three times. Data are presented as means $\pm$ SEM.

was administered to caerulein-induced AP mice *in vivo*. Unfortunately, no obvious histologic alteration of mouse pancreas was observed separately at 12, 18, or 24 hours after treatment with different doses of Nec-1 or Nec-1i (Figure 3A). Neither the serum amylase/lipase activity nor the extent of pancreatic acinar cell necrosis was significantly different in the control group, the Nec-1i group, the Nec-1 low-dose (0.5 mg/kg) group, the Nec-1 middose (1 mg/kg) group, and the Nec-1 high-dose (2 mg/kg) group (Figure 3, B–D).

Treatment with CCK induced ATP depletion in mice pancreatic acinar cells. Co-treatment of Nec-1 at different concentrations did not reverse ATP depletion induced by CCK or L-Arg, which revealed no protective effect of Nec-1

in pancreatic acinar cell necrosis against CCK or L-Arg (Figure 4, A and B). Our results confirm that inhibiting RIP1 kinase activity exerts no protection against caerulein/CCK-induced necrosis *in vivo* and *in vitro*.

RIP1 Inhibits Acinar Cell Necrosis through Activation of NF- κ B *in Vitro*

To further determine the prosurvival or prodeath role of RIP1 in AP necrosis, C57BL/6 mouse pancreatic acinar cells were transfected with RIP1 siRNA, and the survival rate was measured in pancreatic acinar cell after CCK stimulation (Figure 5A). RIP1 inhibition with siRNA transfection failed to inhibit pancreatic acinar cell necrosis

Figure 5 Receptor-interacting protein (RIP) 1 promotes survival through activation of NF- κ B. **A–E**: Primary pancreatic acinar cells were isolated from C57BL/6 mice and were transfected with RIP1 or the control siRNA. Twelve hours after transfection, cells were treated with 200 nmol/L cholecystokinin-8 (CCK) for an additional 7 hours. **A**: Cell lysates were collected and subjected to Western blotting analysis of RIP1 and β -actin to determine the knockdown efficiency of RIP1 siRNA. **B**: Cell viability was determined by measuring ATP levels. **C**: Double staining of Hoechst 33258 and propidium iodide, indicating pancreatic acinar cell necrosis after RIP1 siRNA transfection and CCK stimulation. **Arrows** indicate necrotic cells. The extent of acinar cell necrosis was expressed as a percentage of total cells in 10 randomly chosen fields. **D**: Nuclear extracts were collected and subjected to Western blotting analysis of NF- κ B and histone H₃. **E**: Five micrograms of nuclear extracts were subjected to electrophoretic mobility shift assay using a DNA probe containing NF- κ B binding elements. **F**: Primary pancreatic acinar cells were isolated from C57BL/6 mice and treated with or without 200 nmol/L CCK and 10, 20, and 40 nmol/L Bay11-7085 (NF- κ B inhibitor) for 6 hours. Cell viability was determined by measuring ATP levels. All experiments were performed at least three times. Data are represented as means $\pm$ SEM. * P < 0.05 versus N; † P < 0.05 versus NC-siRNA; ‡ P < 0.05 versus CCK. Original magnification: $\times$ 200 magnification (C). N, without CCK; NC, negative control.

Figure 6 Efficacy of receptor-interacting protein (RIP) 3 and mixed lineage kinase domain-like (MLKL) inhibition on pancreatic acinar cell necrosis. **A–C:** Primary pancreatic acinar cells were isolated from BALB/c mice and transfected with either the RIP3 or the control siRNA. Twelve hours after transfection, cells were treated with 200 nmol/L cholecystokinin-8 (CCK) for an additional 6 hours. **A:** Cell lysates were collected and subjected to Western blotting analysis of RIP3 and β -actin to determine the knockdown efficiency of RIP3 siRNA. **B:** Cell viability was determined by measuring ATP levels. **C:** Double staining of Hoechst 33342 and propidium iodide (PI), indicating pancreatic acinar cell necrosis after RIP3 siRNA transfection and CCK stimulation. **Arrows** indicate necrotic cells. The extent of acinar cell necrosis was expressed as a percentage of total cells in 10 randomly chosen fields. **D–F:** Primary pancreatic acinar cells were isolated from BALB/c mice and transfected with MLKL or the control siRNA. Twelve hours after transfection, cells were treated with 200 nmol/L CCK for an additional 6 hours. **D:** Cell lysates were collected and subjected to Western blotting analysis of MLKL and β -actin to determine the knockdown efficiency of MLKL siRNA. **E:** Cell viability was determined by measuring ATP levels. **F:** Double staining of Hoechst 33342 and PI, indicating pancreatic acinar cell necrosis after MLKL siRNA transfection and CCK stimulation. **Arrows** indicate necrotic cells. The extent of acinar cell necrosis was expressed as a percentage of total cells in 10 randomly chosen fields. **G:** Primary pancreatic acinar cells were isolated from C57BL/6, BALB/c, and Institute of Cancer Research (ICR) mice and were treated with 200 nmol/L CCK for 6 hours. Whole cell extracts were analyzed via Western blotting analysis for phosphorylated MLKL (pMLKL) protein expressions. **H and I:** Primary pancreatic acinar cells were isolated from BALB/c mice and transfected with either the RIP3 or the control siRNA. Twelve hours after transfection, cells were treated with 200 nmol/L CCK for an additional 6 hours. **H:** Cell lysates were collected and subjected to Western blotting analysis of pMLKL and β -actin. **I:** Cell lysates were then collected and subjected to enzyme-linked immunosorbent assay analysis of trypsinogen activation peptide (TAP) concentration. All experiments were performed at least three times. Data are presented as means $\pm$ SEM. * $P < 0.05$ versus N; † $P < 0.05$ versus NC-siRNA. Original magnification: $\times 200$ (C and F). N, without CCK; NC, negative control.

but resulted in greater pancreatic acinar cell necrosis (Figure 5, B and C). Notably, RIP1 inhibition led to approximately 10% necrosis in normal acinar cell (Figure 5B).

Activation of the NF- κ B signaling pathway by RIP1 led to the translocation of NF- κ B to the nucleus and promoted the expression of prosurvival genes, such as cFLIP and c-IAP. To further clarify the possible involvement of NF- κ B activation in exerting a prosurvival role of RIP1 in CCK-stimulated pancreatic acinar cell, we detected the expression of NF- κ B in the nucleus by Western blotting and its transcription activity by electrophoretic mobility shift assays.

RIP1 inhibition led to a reduction of NF- κ B expression in the nucleus (Figure 5D) and decreased NF- κ B transcription activity (Figure 5E). In addition, inhibition of NF- κ B with chemical reagents Bay 11-7085 led to reduced pancreatic acinar cell survival (Figure 5F). To determine the effect of RIP1 in inflammation, we detected the mRNA expression of proinflammatory mediators (TNF- α , IL-6, and IL-1 β) in the cells by RT-PCR and protein expression in the cell culture supernatant by ELISA. Our results indicate that inhibition of RIP1 with siRNA failed to inhibit the production and release of proinflammatory cytokine (Supplemental Figure S2, A and B).

Figure 7 Receptor-interacting protein (RIP) 1 inhibits RIP3 expression in pancreatic acinar cells *in vitro*. **A:** Primary pancreatic acinar cells were isolated from BALB/c mice and treated with 200 nmol/L cholecystokinin-8 (CCK) for 6 hours. Western blotting analysis of RIP1 was detected after immunoprecipitation (IP) by application of an anti-RIP3 antibody. **B:** Primary pancreatic acinar cells were isolated from C57BL/6 mice and transfected with either RIP1 or the control siRNA. Twelve hours after transfection, cells were treated with 200 nmol/L CCK for an additional 6 hours. Cell lysates were collected and subjected to Western blotting analysis of RIP1, RIP3, and β-actin. Representative blots from three independent experiments and band intensities determined by densitometry scanning were presented. **C:** Primary pancreatic acinar cells were isolated from C57BL/6 mice and treated with 200 nmol/L CCK and necrostatin-1 (Nec-1) (10, 30, and 60 μmol/L) for 6 hours. Cell lysates were collected and subjected to Western blotting analysis of RIP3 and β-actin. Representative blots from three independent experiments and band intensities determined by densitometry scanning were presented. All experiments were performed at least three times. Data are presented as means ± SEM. **P* < 0.05 versus N; †*P* < 0.05 versus NC-siRNA. N, without CCK; NC, negative control.

RIP3 Promotes Acinar Cell Necrosis through Regulation of pMLKL but Independent of Trypsinogen Activation

Previous reports have found that genetically knocked-out RIP3 mice had less severe AP after caerulein injection.^{14,15} Our results indicate that inhibition of RIP3 with RIP3 siRNA partially rescued mouse pancreatic acinar cell necrosis induced by CCK *in vitro* (Figure 6, A–C). Similarly, inhibition of MLKL with MLKL siRNA partially rescued mice from pancreatic acinar cell necrosis induced by CCK *in vitro* (Figure 6, D–F).

To further confirm the presence of necroptosis in AP and acinar cells, antibodies were used to specifically detect pMLKL in pancreatic acinar cells of three mice species. The expression of pMLKL in pancreatic acinar cells after CCK stimulation was significantly elevated in BALB/c and ICR mice, but there was no significant change in C57BL/6 mice (Figure 6G). This finding is in accordance with the significant elevation of necrosis in BALB/c and ICR mice and the absence of robust elevation in C57BL/6 mice detected by the ATP assay (Figure 1H). The expression pattern further confirmed the correlation between necroptosis and AP severity.

RIP3 has been found to phosphorylate and activate MLKL, which executes cell necroptosis. To further illustrate the association between RIP3 and MLKL in mediating necroptosis, we determined pMLKL expression after RIP3 inhibition. Our results indicate that inhibition of RIP3 with RIP3 siRNA led to lower pMLKL levels; however, MLKL expression was not inhibited (Figure 6H). In summary, our

results indicate that CCK induced RIP3 up-regulation, which exerts necroptosis through phosphorylation of MLKL.

RIP3 and trypsin are two key molecules in mediating pancreatic necrosis, but the association of necroptosis and trypsinogen activation is not determined in AP. To illustrate the possible link between these two factors, we detected trypsinogen activation by ELISA, detecting TAP level after RIP3 inhibition. RIP3 inhibition led to no significant reduction of TAP protein level (Figure 6I), which suggested that RIP3-mediated necroptosis is not dependent on trypsinogen activation.

RIP1 Inhibits RIP3 Expression in Pancreatic Acinar Cells *in Vitro*

As mentioned above, RIP1 inhibits RIP3-mediated necroptosis in acinar cells. To test whether RIP1 and RIP3 interact with each other after necrotic stimuli to form the necrosome, we prepared the whole endogenous proteins from mice CCK-stimulated pancreatic acinar cells, followed by immunoprecipitation by application of an anti-RIP3 antibody. However, no interaction of RIP3 with RIP1 was observed, indicating that RIP1 and RIP3 would not directly interact with each other in necrotic pancreatic acinar cell after hyperstimulation of CCK (Figure 7A). Interestingly, Western blotting analysis revealed that RIP1 inhibition with RIP1 siRNA led to enhanced RIP3 expression (Figure 7B), and inhibition of RIP1 kinase activity had no effect on RIP3 expression (Figure 7C).

Figure 8 Receptor-interacting protein (RIP) 3-dependent necroptosis mediates inflammation in acute pancreatitis (AP). **A–C:** Primary pancreatic acinar cells were isolated from BALB/c mice and transfected with the RIP3 or control siRNA. Twelve hours after transfection, the cells were treated with 200 nmol/L cholecystikinin-8 (CCK) for an additional 6 hours. **A:** Cell lysates were collected and subjected to an enzyme-linked immunosorbent assay test of cleaved IL-1 β protein production. **B:** Cell lysates were collected and subjected to Western blotting analysis detecting pro-IL-1 β and cleaved IL-1 β expression. **C:** Cell lysates were collected and subjected to Western blotting analysis detecting NLRP3, pro-caspase-1, and cleaved caspase-1 expression. All experiments were performed at least three times. Data are presented as means $\pm$ SEM. * $P < 0.05$ versus N; † $P < 0.05$ versus NC-siRNA. N, without CCK; NC, negative control.

RIP3-Dependent Necroptosis Mediates Inflammation in AP

Necrosis and inflammation are two key pathologic processes of AP. Inflammasome activation, which mediates IL-1 β maturation, is involved in the pathogenesis of AP. To illustrate whether RIP3-mediated necroptosis participates in inflammasome activation in AP, we determined IL-1 β expression by Western blotting and the mature form cleaved IL-1 β expression by ELISA analysis. Our results indicate that the expression of cleaved IL-1 β was significantly inhibited by RIP3 inhibition, whereas pro-IL-1 β was not inhibited (Figure 8, A and B). Furthermore, inhibition of RIP3 led to reduced NLRP3 expression and caspase-1 cleavage (Figure 8C). Our results indicate that RIP3 mediates NLRP3 inflammasome activation and IL-1 β maturation.

Discussion

We developed two experimental models of AP induced by the same caerulein treatment in rats and mice. The extent of apoptosis peaked at 9 hours but later decreased, indicating that apoptosis acts as a self-restricted pattern of cell death. On the contrary, necrosis proceeded in an uncontrolled manner because the extent of necrosis progressed with time. The natural progression attributable to the phagocytosis properties by inflammatory cells and the abilities to trigger inflammation between the two modes of cell death make apoptosis a protective response and necrosis a deleterious response in human disease.²⁶ The rat model had predominant apoptosis and presents as mild disease, whereas the mouse model had predominant necrosis and presents as severe disease. The cell death patterns in AP revealed that the severity of AP directly correlates with necrosis and inversely correlates with apoptosis, which is in accordance

with previous results.⁴ Furthermore, three different severities of the murine AP model were established *in vivo* and *in vitro*. We found that ICR mice with greatest susceptibility to AP had the most severity of necrosis in caerulein-induced AP mice and CCK-stimulated pancreatic acinar cells, BALB/c mice with modest severity had less necrosis, and C57bl/6 mice with mild severity had the least necrosis. These results further indicate that the degree of necrosis determines AP severity. Therefore, determining how a cell succumbs to apoptosis and not necrosis may guide the development of new therapeutic strategies for AP.

Necroptosis, a newly discovered form of necrosis dependent on RIP3, is regulated by specific genes and has the typical morphologic features of necrosis. Genetic knockout of RIP3 or MLKL ameliorates the severity of caerulein-induced AP in mice,^{14,15,27} which confirmed the existence of necroptosis in AP. RIP3 executes necroptosis in response to several stimuli under caspase compromised conditions.^{14,15,27} Tissue-specific deletion of caspase-8 causes disease that involves cell death and can be prevented by the ablation of RIP3,¹³ indicating a critical function of caspase-8 in the prevention of RIP3-mediated necroptosis. In the rat model, caspase activation was observed, which mediates apoptosis, whereas in the mouse model, caspase activation was inhibited, which facilitates RIP3-mediated necroptosis. Caspase activation inhibits RIP3-mediated necroptosis through the cleavage of RIP3.^{28,29} As a result, we believe that it is possible to therapeutically interfere with necrosis in AP.

Although some studies indicate the involvement of necroptosis in the development of AP, the underlying molecular mechanisms are still not clear. The *in vivo* and *in vitro* studies found that RIP3 protein expression in mouse pancreas tissue and pancreatic acinar cell was significantly elevated after caerulein/CCK stimulation. The up-regulation of RIP3 could be a stress reaction of pancreatic acinar cell to the stimulus or as a result of infiltration inflammatory cells. The

induction of RIP3 observed in a number of pathologic conditions is likely to be the mechanism underlying increased necroptosis in the pathogenesis of these diseases.^{12,30} Genetic knockout of RIP3 ameliorates the severity of caerulein-induced AP in mice,^{14,15} which revealed that induction of RIP3 was responsible for necroptosis. Our *in vitro* studies found that knockdown of RIP3 with siRNA partly alleviated CCK-induced pancreatic acinar cell necrosis. The results also indicate that RIP3-dependent necroptosis is involved in caerulein/CCK-induced acinar cell injury. In accordance with the critical role of RIP3 in necroptosis, RIP3 protein expression was positively related to necrosis in caerulein-induced AP mice and CCK-stimulated acinar cells in different species of mice.

A critical question concerns the nature of the downstream events that are triggered by RIP3 activation, which may execute necrotic cell death. Genetic study provides evidence of the essential role of MLKL in AP-related necroptosis because knockout of MLKL ameliorates the severity of caerulein-induced AP in mice.²⁷ Our *in vitro* results indicate that knockdown of MLKL with siRNA partly reversed necrosis in CCK-stimulated pancreatic acinar cells, which is in accordance with results in MLKL-deficient mice *in vivo*.²⁷ RIP3-mediated phosphorylation of MLKL drives oligomerization of MLKL, which elicits MLKL translocation to membranes to mediate necrotic cell death.^{31,32} RIP3 inhibition decreased CCK-induced MLKL phosphorylation, whereas the expression of MLKL was not significantly changed in acinar cells. These results indicate that RIP3 mediates necroptosis through MLKL phosphorylation in acinar cells. The expression of pMLKL was up-regulated most significantly in ICR mice, less significantly in BALB/c mice, and least significantly in ICR mice. The consistent association between AP severity and pMLKL expression makes pMLKL an ideal marker for detecting necroptosis and predicting AP disease severity.

RIP1 is a pleiotropic protein that can determine cell death or cell survival. RIP1 can activate NF- κ B, leading to cell survival, or associate with RIP3, leading to apoptosis or necroptosis. RIP1 kinase activity plays a significant role in mediating necroptosis because mice genetically expressing kinase inactive RIP1 were unable to mediate necroptosis. Particular cell types may require RIP1 for necroptosis,^{12,33} although whether pancreatic acinar cell necroptosis requires RIP1 during AP remains unclear. Evidence of the requirement of RIP1 in necroptosis can be gained in two ways: through the use of RIP1 kinase inhibitor or through knockdown and gene inactivation of RIP1. Our *in vivo* and *in vitro* results indicate that RIP1 kinase inhibitor Nec-1 did not protect against necrosis in AP mice and CCK-stimulated pancreatic acinar cells. We cannot completely rule out the possibility that these negative results could be attributable to a lack of targeting *in vivo*. However, knockdown of RIP1 with siRNA further aggravated CCK-induced necrosis. Interestingly, we also found that knockdown of RIP1 resulted in approximately 10% to 20% of acinar cell spontaneous necrosis. Taking into account this fact, we

propose that RIP1 acts as an intrinsic prosurvival factor in normal acinar cells. A major difference between the inhibition of RIP1 kinase activity using Nec-1 and the knockdown of RIP1 is that Nec-1 only impedes RIP1-dependent necroptosis but not RIP1-dependent NF- κ B activation, whereas the knockdown of RIP1 inhibits both RIP1-dependent necroptosis and NF- κ B activation. These data indicate that RIP1 mediates acinar cell survival. Moreover, RIP1 expression was negatively related to necrosis in caerulein-induced AP mice and CCK-stimulated pancreatic acinar cells. These results suggest that RIP1 could have antinecrosis effect in the setting of AP and challenge the current definition of necroptosis.

Furthermore, we found that RIP1 down-regulation was in accordance with decreased expression of NF- κ B in the nuclear extracts and reduced transcription activity of NF- κ B. NF- κ B mediates the expression of genes that play key roles in inflammation and cell survival in AP.³⁴ Although the mechanisms are still unclear, two studies revealed the role of NF- κ B in protecting against acinar cell necrosis, which can limit pancreatic inflammation.^{35,36} In our study,

Figure 9 Mechanisms of receptor-interacting protein (RIP) 1 and RIP3 in mediating necrosis and inflammation in experimental acute pancreatitis (AP). MAP, mild acute pancreatitis; pMLKL, phosphorylated mixed lineage kinase domain-like; SAP, severe acute pancreatitis.

pharmacologic NF- κ B inhibition aggravated pancreatic acinar cell necrosis induced by CCK stimulation *in vitro*. These results indicated that RIP1 promotes pancreatic acinar cells survival, possibly through NF- κ B activation.

In the early studies of necroptosis, two serine/threonine kinases, RIP1 and RIP3, were identified as the key regulators of necroptosis.^{12,21} RIP1 expression was negatively related to AP disease severity, RIP3 expression was positively related to AP disease severity, and the expression of these two proteins were negatively related. These findings prompted us to further clarify the possible association between the two proteins. We did not observe the components of RIP1/RIP3 in CCK-induced pancreatic acinar cell necrosis, and the knockdown of RIP1 led to enhanced RIP3 expression in acinar cells. These results suggest that RIP3-dependent, but not RIP1, necroptosis contributes to pancreatic damage in experimental pancreatitis. On the contrary, RIP1 exerts protective effects by inhibiting RIP3-mediated necroptosis on experimental pancreatitis.

Pancreatic acinar cells stimulated with CCK led to more production of mature IL-1 β . IL-1 β production is associated with two signals; one is transcriptional regulation by NF- κ B, which induces pro-IL-1 β transcriptional induction, and the other is inflammasome activation, which cleaves pro-IL-1 β , leading to IL-1 β maturation. NLPR3-mediated inflammasome activation was required for inflammation in AP.³⁷ In addition to mediating necroptosis, RIP3 can also promote caspase 1-mediated pro-IL-1 β processing. However, there is disagreement on whether the enhanced IL-1 β production was attributable to increased necrosis-associated release of damage-associated molecular patterns or direct effects of RIP3 on caspase-1 activation. Regardless of the mechanism, it is clear that RIP3 can promote caspase-1-mediated pro-IL-1 β processing. RIP3 inhibition led to less IL-1 β activation and less NLPR3-mediated caspase-1 activation, thereby indicating that RIP3 was involved in NLPR3-mediated inflammasome activation in CCK-stimulated pancreatic acinar cells.³⁸ Intracellular trypsinogen activation has been suggested as a primary mechanism in mediating necrosis in AP. Our results found that RIP3 inhibition did not affect TAP protein level, which suggests that RIP3-mediated necroptosis is not dependent on trypsinogen activation.

In summary, our study strongly suggests that RIP3 contributes to acinar cell necrosis by phosphorylating MKLK, whereas RIP1 protects against acinar cell necrosis possibly through activating the NF- κ B signaling pathway in experimental pancreatitis (Figure 9). Therefore, therapeutic approaches that enhance RIP1 expression or inhibit RIP3 expression may provide a novel strategy for preventing or decreasing necrosis in pancreatitis.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2016.12.021>.

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